EXERCISE INDUCED NEUROPROTECTION IN SPONTANEUOSLY RUNNING RATS.

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PREFACE

Parkinson's disease is a disease of the basal ganglia dopamine neurons that is characterized by a progressive loss of more than 70% of the dopaminergic neurons in the substantia nigra. Treatments that have been used to alleviate the symptoms of Parkinson's disease include the use of pharmacological drugs that are direct or indirect agonists of dopamine release and electric stimulation of the subthalamic nucleus. However the use of pharmacological drugs can lead to unbearable side effects while subthalamic nucleus stimulation or lesion requires surgery and is thus invasive. It has also been shown that as the disease progresses, the pharmalogical drugs lose their efficacy. Neurotoxic drugs such as 6-hydroxydopamine have been used to mimic a parkinsonian state in a rat model resulting in a dopamine neuron deficit in the lesioned hemisphere. In unilaterally lesioned rats, the toxic effect of 6-hydroxydopamine has been shown to be reduced in the striatum of rats that were forced to exercise. However forced exercise may not be ideal in treating Parkinson's disease patients. This then raises the question of whether voluntary exercise has the same beneficial effects on dopamine neuron survival as forced exercise following 6-hydroxydopamine lesion, whether these beneficial effects if any are due to the neuroprotective properties of neurotrophins and finally whether perinatal stress and stress in adulthood results in the reversal of the beneficial effects of exercise.

ABSTRACT

Exercise induced neuroprotection in spontaneously running rats
Musa Vuyisile Mabandla. March 2007

Study 1: We investigated the effects of voluntary exercise on neuroprotection after unilateral lesions with 6-hydroxydopamine. Rats were divided into runners (had access to running wheel) and non-runners (their running wheels were immobilised). Two weeks after injection of the neurotoxin, the rats were injected with apomorphine and the number of ipsilateral and contralateral rotations was counted with contralateral rotations of greater than 150 considered to represent striatal dopamine neuron destruction of 70% or above. The number of contralateral turns made by the non-runners was significantly greater than the number of turns made by the non-runners. To confirm our results we counted the number of dopamine neurons present in the lesioned and non-lesioned hemispheres and the percentage of dopamine remaining in the lesioned striatum of the rats using tyrosine hydroxylase immunohistochemistry and high performance liquid chromatography respectively. We found that exercise provides neuroprotection as the percentage of dopamine destruction was significantly less in the lesioned substantia nigra of the runners than in the lesioned substantia nigra of the non-runners. The percentage of striatal dopamine remaining in the lesioned hemisphere was greater that 70% and less than 30% in the runners and non-runners respectively.

Study 2: We investigated the effect of stress on lesioned rats that had access to running wheels (stressed runners) and compared them to runners and non-runners. We measure plasma adrenocorticotrophic hormone levels, corticosterone levels using radioimmunoassays, and glial cell-line derived neurotrophic factor concentration in the striatum, substantia nigra and ventral tegmental area using ELISA. We found that the basal corticosterone levels in the runners and stressed runners were significantly greater than the basal corticosterone levels in the non-runners. However only the basal ACTH levels of the runners were significantly elevated. There was no significant difference between the glial cell-line derived neurotrophic factor concentration in the

lesioned hemisphere of the three groups. However the glial cell-line derived neurotrophic factor concentration in the non-lesioned substantia nigra of the stressed runners was significantly less than in the non-lesioned substantia of the non-runners.

Study 3: We investigated whether the expression of glial-cell-line derived neurotrophic factor is increased in exercising rats that have not been lesioned. We also looked at whether exercise attenuates the stress response following acute restraint stress. We found that post restraint stress corticosterone levels were significantly raised from baseline levels in the runners whereas the corticosterone response to acute restraint stress was minimal in the non-runners. There was no significant difference in glial cell-line derived neurotrophic factor concentration between the runners and the non-runners. Exercise did not increase glial cell-line derived neurotrophic factor expression in non-lesioned rats.

Study 4: We investigated whether adult offspring of rats that received various stressors during gestation have the same abnormal behavioural and neuroendocrine effects as other models of prenatal stress. We had three groups of rats, rats that received food and water *ad libitum*, rats that were 50% food deprived in the third week of gestation and rats that received various stressors also in the third week of gestation. Behavioral tests which included the open field test and the elevated plus maze test were performed on adult offspring and trunk blood was collected for basal and post restraint neuroendocrine measurements. There was no significant difference between the time the three groups spent in the closed and open arms of the elevated plus maze. The distance covered by the stressed rats in the open field was significantly less than the distance covered by the non-stressed rats. There was no significant difference between the weights. The weight of the adrenal glands was also not significantly different. The corticosterone response to stress was significantly elevated in all the rats. However there was a blunted ACTH response to stress in the stressed rats.

Study 5: We investigated the effects of exercise on adult offspring of rats that were prenatally stressed following lesion with 6-hydroxydopamine. Prenatally stressed and non-stressed rats were divided into non-runners and runners. Behavioral tests performed included the step test, cylinder test, and the open field test. Rats were

sacrificed and dopamine neuron degeneration was counted in the substantia nigra using tyrosine hydroxylase immunohistochemistry. Dopamine neurone destruction was significantly greater in prenatally stressed rats than in the non-stressed runners. There was also increased limb use asymmetry in the prenatally stressed runners than in the non-stressed runners suggesting that exercise did not provide neuroprotection in the prenatally stressed rats.

Study 6: We investigated whether exercise following 6-hydroxydopamine injection has the same effect in adult rats that were maternally separated as in adult offspring of rats that were prenatally stressed. There was a significant difference between the non-stressed runners and the other rats in limb use asymmetry suggesting motor function impairment. However there was no significant difference between the maternally separated runners and the non-stressed runners in the percentage dopamine neurone destruction in the substantia nigra suggesting that exercise provided neuroprotection in the maternally separated rats which was not found in the prenatally stressed rats.

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
AVP	Arginine vasopressin
cm	Centimeter
CORT	Corticosterone
cCREB	cyclic AMP response element binding protein
CRF/H	Corticosterone releasing factor/hormone
DNA	Deoxyribonucleic acid
DA	Dopamine
DAB	Diaminobenzidine tetrahydrochloride
DAT	Dopamine transporter
D1	Dopamine receptor subtype 1
D2	Dopamine receptor subtype 2
BDNF	Brain derived neurotrophic factor
E	Gestational day
EDTA	Ethylenediamine tetra-acetic acid
GABA	Garnma-aminobutyric acid
GDNF	Glial cell-line derived neurotrophic factor
GRF α	Glial cell-line neurotrophic factor family receptor alpha
GPe	Globus pallidus externa/ external globus pallidus
GPi	Globus pallidus interna/ internal Globus pallidus
g	Gram
HCl	Hydrochloric acid
h	Hour
HPA axis	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
11ß-HSB	11ß-hydroxysteroid dehydrogenase
ip	Intraperitoneal
KCl	Potassium chloride

Kg	kilogram
KH2PO4	Potassium dihydrophosphate
MAP-K	Mitogen activated protein kinase
MAO	Monoamine oxidase
MFB	Medial forebrain bundle
MPTP	1-methy-4-phenyl-1,2,3,6 tetrahydropyridine
m	meter
mg	Milligram
ml	Milliliter
mm	Millimeter
Min	Minutes
M	Molar
MSNR	Maternally separated non-runner
MSR	Maternally separated runner
Na2HPO4	di-sodium hydrophosphate
NaOH	Sodium hydroxide
ng	Nanogram
NGF	Nerve growth factor
NR	Non-runner
NSNR	Non-stressed non-runners
NSR	Non-stressed runner
6-OHDA	6-Hydoxydopamine
P	Postnatal day
PBS	Phosphate buffered saline
PKC	Protein kinase C
PVN	Paraventricular nucleus
sc	Subcutaneous
sec	Seconds
SNR	Stressed non-runners
SNc	Substantia nigra pars reticulata
SNr	Substantia nigra pars compacta

SR	Stressed runner
SNT	Subthalamic nucleus
R	Runner
ROS	Reactive oxidative stress
TC	Total counts
TH ICC	Tyrosine hydroxylase immunohistochemistry
Trk	Tyrosine receptor kinase
µg	Microgram
µl	Microliter
mRNA	Messenger ribonucleic acid
mV	Millivolts
VTA	Ventral tegmental area

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PUBLICATIONS

1. Mabandla, M., Kellaway. L., St Clair Gibson, A., Russell V. (2004). Voluntary running provides neuroprotection in rats after 6-OHDA injection into the medial forebrain bundle. Met Brain Res **19:** 43-50
2. Howells, F.M., Russell, V.A., Mabandla. M.V., Kellaway, L.A. (2005). Stress induces the neuroprotective effect of exercise in a rat model for Parkinson's disease. Behav.Brain.Res **165:** 210-220.
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CHAPTER 1

Literature Review

1.1 The Basal ganglia

The basal ganglia comprise a set of subcortical nuclei that are critical in the control of motor movements *(Awad et al 2000)*. These subcortical nuclei are the striatum, substantia nigra, globus pallidus and subthalamic nuclei *(Table 1.1.1, Kandell et al 2000)*. The striatum consists of two subdivisions, the caudate putamen and the nucleus accumbens which is part of the ventral striatum whereas the substantia nigra consists of the pars reticulata and pars compacta and the globus pallidus has an external and internal subdivision *(Kandell et al 2000, Squire et al 2003)*. In the basal ganglia, neuronal inputs from the cortex are processed and then relayed to areas of the brain stem and motor cortices that are involved in planning and production of movement *(Kandell et al 2000, Squire et al 2003)*. The neuronal network of the basal ganglia includes a dopaminergic neuron network that projects from the substantia nigra pars compacta to the striatum and projections from the striatum to the substantia nigra that consist of GABAergic neurons *(Ganong et al 2005)*. For this reason, the striatum is viewed as the primary input structure of the basal ganglia with the internal globus pallidus (GPi) and the substantia nigra pars reticulata (SNr) being the main output structures *(Wichmann et al 2003)*. The input and output structures of the basal ganglia are linked by a monosynaptic (direct) pathway and a polysynaptic (indirect) pathway that involves the external globus pallidus and the subthalamic nucleus *(Kandell et al 2000, Squires et al 2003, Wichmann et al 2003)*. The subthalamic nucleus consists of excitatory glutamatergic neurons that provide excitatory inputs to the substantia nigra pars reticulata and internal globus pallidus (output structures) *(Awad et al 2000)*. Dopamine in the striatum enhances the transmission of neuronal signals along the direct pathway by using the dopamine receptor subtype 1 (D1 receptors) and reduces transmission in the

indirect pathway by utilizing the dopamine receptor subtype 2 (D2 receptors) *(Wichmann et al 2003)*.

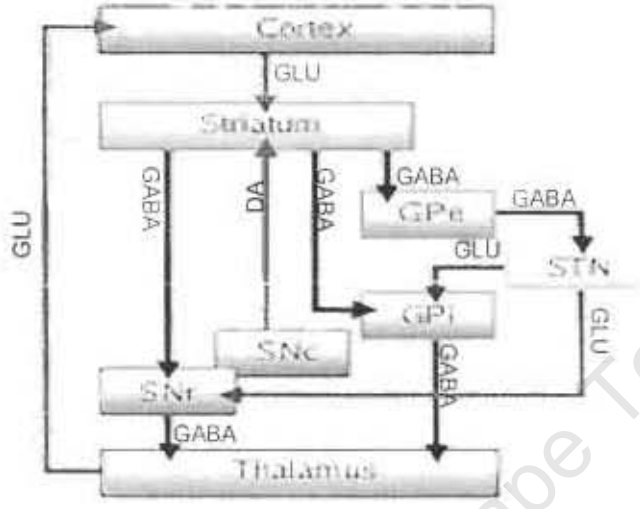


Figure 1.1.1 Schematic representation of the interconnections of the neurons in the cortex, basal ganglia, thalamus, subthalamic nucleus (SNT), substantia nigra pars reticulata and compacta (SNr and SNc respectively) and the globus pallidus interna and externa (GPi and GPe respectively). The neurotransmitters utilized in these pathways include glutamine (GLU), y-aminobutyric acid (GABA) and dopamine (DA).

The dopamine neurons in the nigrostriatal pathway consists of the A9 cell group that is located in the substantia nigra pars compacta which has axons that run along the medial forebrain bundle (MFB) and terminate in the dorsal striatum *(Deumens et al 2002, Fallon et al 1978)*. Dopamine neurons release dopamine in the somatodendritic level of the substantia nigra pars compacta as well as in the terminal regions in the striatum *(Sarre et al 2004)*. The somatodendritic release of dopamine is important in the control of movement as the substantia nigra pars

reticulata and the internal globus pallidus form the main outputs from the basal ganglia and hence are important in the control of movement *(Albin et al 1989, Kandell et al 2000, Squires et al 2003, Sarre et al 2004)*. Somatodendritic dopamine release targets D2 receptors on the neuron cell bodies as well as D1 receptors in the GABAergic striatonigral afferents *(Sarre et al 2004)*. For normal motor function, an intricate balance between the excitatory effects provided by the glutaminergic neurons in the subthalamic nucleus on the substantia nigra pars reticulata, the internal globus pallidus and the GABAergic projections from the striatum needs to be maintained *(Awad et al 2000)*. Dopamine is formed by the conversion of the amino acid tyrosine by the enzymes tyrosine hydroxylase and aromatic amino acid decarboxylase *(Zigmond et al 1990)*. When dopamine is released by the terminals, some of the dopamine diffuses to distant sites where it gets removed quickly from the synaptic cleft by the dopamine transporter (DAT) and is inactivated by the enzyme monoamine oxidase (MAO) (*Zigmond et al 1990*).

1.2 Parkinson's disease

Degeneration of the dopaminergic neurons in the nigrostriatal pathway leads to a disease of the basal ganglia called Parkinson's disease *(Kandell et al 2000, Squire et al 2003, Ganong et al 2005)*. The nigrostriatal pathway is able to compensate for dopamine cell loss and only when more than 80% of the dopamine neurons in the substantia nigra have degenerated are the symptoms of Parkinson's disease present *(Offen et al 2001)*. It is the compensatory response of the surviving dopamine neurons and the post synaptic cells in the striatum that help mitigate the progressive loss of dopamine innervation *(Deumens et al 2002)*. The compensatory response in the surviving cells includes an increase in the activity of the remaining dopamine cells that leads to an increase in dopamine release resulting in an increase in metabolic turnover and an increase in post synaptic dopamine receptor density and/or sensitivity *(Deumens et al 2002)*. For instance when there is partial degeneration of the

presynaptic terminals, the functional terminals increase dopamine biosynthesis by increasing the rate of deamination and the amount of tyrosine hydroxylase protein available *(Zigmond et al 1990)*.

When dopamine is released from the terminal, some may move to a distant synapse and because this synapse has degenerated, the dopamine is not removed by DAT and thus can bind to the postsynaptic receptors present in the area *(Zigmond et al 1990)*. It is the dopaminergic projections from the substantia nigra pars compacta to the putamen of the striatum that are severely affected *(Squires et al 2003, Ganong et al 2005)*. Extensive loss of dopamine in the A9 cells leads to a dramatic decline in striatal dopamine and external globus pallidus activity *(Deumens et al 2002, Wichmann et al 2003)*. Studies have shown that there is a significant increase in dopamine D1 and D2 receptor function in the striatum of patients with Parkinson's disease patients *(Seemans et al 1996)*. Studies have also suggested that the loss of nigrostriatal dopamine results in an increase in the firing of the excitatory neurons of the subthalamic nucleus that leads to a concomitant increase in the firing of the inhibitory GABAergic neurons to the output neurons of the basal ganglia *(Wichmann et al 1997, DeLong 1990)*. This result in decreased firing of the thalamocortical projections which leads to the impaired (hypokinetic) motor movements associated with Parkinson's disease *(DeLong 1990)*.

The symptoms of Parkinson's disease include a tremor at rest that disappears during movement, slowness of movement (bradykinesia), paucity of movement (akinesia), muscular rigidity and unstable posture *(Squires et al 2003)*.
One of the causes of the neurodegeneration that eventually results in Parkinsonism is thought to be an increase in oxidative stress *(Cohen et al 1974, Zigmond (b) et al 2002)*. Analysis of the brains of Parkinson's disease patients postmortem has shown an increase in superoxide dismutase and other changes commonly associated with reactive oxidative stress such as an increase in oxidative damage to proteins and DNA *(Olanow et al 1999)*. One of the more

reactive oxidative stress (ROS) analogues is 6-OHDA, a compound that is neurotoxic to both dopamine and norepinephrine neurons *(Tillerson et al 2001, Zigmond(b) et al 2002)*. A Parkinsonian animal model can be created by injecting the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice *(Yuan et al 2005)*. However the disadvantage in using MPTP is the acute nature of its toxicity whereas Parkinson's disease is progressive *(Yuan et al 2005)*. A 6-OHDA model is advantageous as the motor deficits that develop are easily quantifiable using pharmacological agents such as amphetamine and apomorphine that have effects on dopamine and its receptors *(Deumens et al 2002, Ungerstedt 1971)*.

1.3 6-Hydroxydopamine (6-OHDA).

Neurotoxic drugs such as 6-OHDA, have made it possible to create a Parkinsonian state in a rat model *(Ungerstedt 1971)*. 6-OHDA is specific to catecholamine neurons such as dopamine and norepinephrine neurons and when it is transported into the cell bodies and fibres of these neurons, it causes their degeneration *(Tillerson et al 2001)*. This occurs because 6-OHDA undergoes auto-oxidation when it enters a dopamine neuron *(Cohen et al 1974, Kearns et al 1997)*, resulting in the formation of highly reactive oxygen species such as superoxide radical, hydroxyl radical and hydrogen peroxide *(Cohen et al 1974)*. The neurotoxicity of these reactive oxygen species is based on their ability to inhibit mitochondrial respiratory enzymes (electron transport chain complexes 1 and 2) by facilitating lipid peroxidation and nucleic acid and protein degradation *(Kearns et al 1997, Deumens et al 2002)*. The metabolic deficiencies that occur as a result of the blockade of this enzyme chain results in the neurons failing to perform normal physiological functions leading to neuronal death *(Glinka et al 1997)*. Injecting 6-OHDA into the medial forebrain bundle *(Table 1.1.2)* of a rat, leads to rapid cell death and has been shown to create a severe lesion that mimics end stage Parkinson's disease *(Ungerstedt, 1971, Yuan et al 2005)*. *Lee et al (1996)* argues that the onset of Parkinson's disease is gradual as evidenced

by the presence of many atrophic neurons in the substantia nigra *(Mann et al 1983)* therefore for neuroprotective studies, they have suggest that injections of 6-OHDA should be made into the striatum where retrograde degeneration of the dopaminergic neurons is gradual and protracted when compared to the acute onset associated with medial forebrain bundle (MFB) lesions *(Lee at al 1996)*.

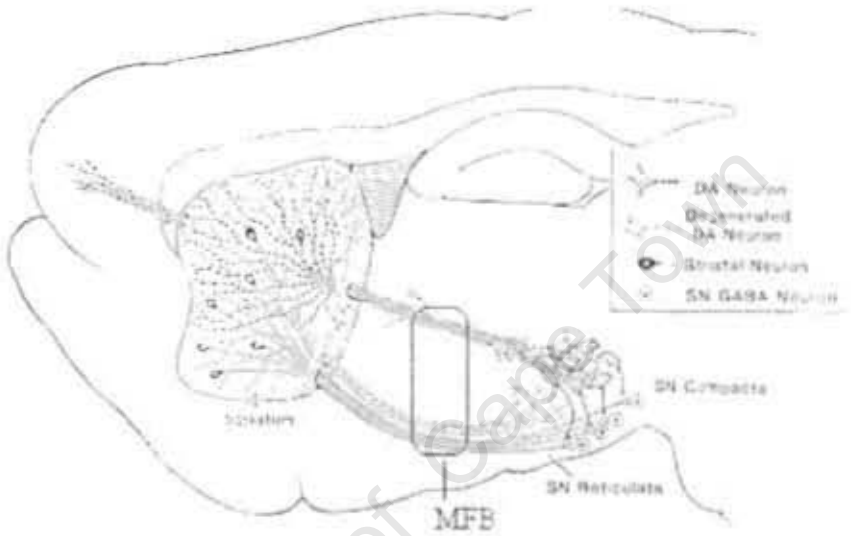


Figure 1.1.2 Schematic representation of the location of the MFB with respect to the substantia nigra and striatum *(Adapted from Zhou et al;(1996) J. Neurosci 16(21))*.

However studies that involved injecting 6-OHDA into the MFB have shown that by manipulating the impaired limb to exercise immediately after lesioning, neuroprotection can be achieved *(Tillerson et al 2001, Tillerson et al 2002)*. Depletion of dopamine concentration in the striatum is reported to be maximal 5 to 7 days after 6-OHDA infusion into the MFB *(Smith et al 2002)*. In our studies, we wanted to look at the effects of exercise in a Parkinsonian rat model hence we preferred to inject 6-OHDA into the MFB rather than in the striatum.

1.4. Exercise

Physical activity has been shown to improve motor function in Parkinson's disease patients *(Hirsch 2000, Toole et al 2000)*. It has also been suggested that physical activity early in life may protect against the development of Parkinson's disease *(Brasted et al 1999)*. Rats that are forced to exercise on a treadmill have been shown to have increased angiogenesis and astrocyte concentration in the cortex and striatum *(Li et al 2005)*. An increase in blood supply promotes an increased delivery of oxygen and glucose to active neurons *(Vissing et al 1996)*. An increase in energy can facilitate protein synthesis which has been shown to be necessary for neuroprotection in injured or damaged tissue *(Kearns et al 1997)*. Astrocytes have been shown to be capable of regulating neurogenesis in the hippocamus by instructing stem cells to differentiate into neurons *(Stevens et al 2002)*. Astrocytes also regulate synaptic formation and transmission *(Stevens et al 2002)*. Treadmill running has been shown to be neuroprotective in vascular injuries such as strokes *(Ding et al 2004)*. On the other hand voluntary wheel running has been shown to upregulate proteins involved in synaptic trafficking, signal transduction pathways, gene transcription regulators, neurotrophs and neurotransmitters in the rat hippocampus *(Molteni et al 2002)*. One such neurotrophic factor is brain derived neurotrophic factor (BDNF) *(Neeper et al 1996)* (SECTION 1.5.1). A symptom of Parkinson's disease that occurs before a clinical diagnosis is made is a decrease in physical activity *(Tillerson et al 2002)*. By injecting a dose of 6-OHDA that is sufficient enough to create 20% nigrostriatal dopamine neuron destruction, it has been shown that when the rat was forced not to use the impaired limb, the neuron destruction grew to almost 60% seven days later *(Tillerson et al 2002)*. When rats were forced to exercise the impaired forelimb immediately following a unilateral 6-OHDA injection into the MFB, it was found that there was complete sparing of the dopamine neurons in the substantia nigra of the lesioned side *(Tillerson et al 2001)*. However there appears to be a critical period since if there is a delay in using the impaired limb (3 or 7 days) there was only partial sparing or complete loss of the dopamine

neurons respectively *(Tillerson et al 2001)*. This suggests that a decrease in physical activity is not only a symptom of Parkinson's disease but also exacerbates neuronal destruction *(Tillerson et al 2002)*. Studies have shown that motor enrichment through voluntary exercise, treadmill running skills training or forced limb use promote brain plasticity and functional change *(Kleim et al 2003)*. This results in the brain responding to injury by upregulating trophic factors such as GDNF and BDNF (SECTION 1.51) *(Kleim et al 2003)*. However if the exercise paradigm is stopped before lesioning or after the onset of brain injury, the expression of neurotrophic factors may plummet below baseline resulting in the brain being more vulnerable to the toxic effects of 6-OHDA *(Kleim et al 2003, Tillerson et al 2002.) Tillerson et al (2001)* has also shown that forced use of the impaired limb immediately following a neurotoxic insult to dopamine neurons, results in sparing of the dopamine neurons in the nigrostriatal region of the lesioned hemisphere however this neuroprotective effect of forced use is not permanent becauses when these rats were subsequently forced not to use the impaired limb, it produced severe dopamine terminal loss and functional deficits *(Tillerson et al 2002)*.

Following prolonged running, the hippocampus has increased expression of BDNF *(Neeper et al 1996, Windenfalk et al 1999)*. Some circulating neurotrophic peptides such as Insulin-like growth factor 1 (IGF-1) that are produced outside the brain are known to cross the blood brain barrier and their levels are found to be increased in the hippocampus following physical exercise *(Carro et al., 2000; 2001)*. Exercise-induced secretion of these intra- and extra- brain neurotrophic peptides has been shown to have neurogenic and neuroprotective effects in the dentate gyrus of the hippocampus *(Alberch et al 2002, Fernandez et al 1998, Trejo et al 2001, Åberg et al 2000, van Praag et al 1999)*. Voluntary exercise increases BDNF mRNA in normal rats *(Molteni et al 2000)*. Expression of neurotrophic factors is not limited to exercise since the expression of neurotrophic factors is increased following brain injury in sedentary rats *(Klein et al 2003, Ploughman et al 2005)*.

1.5 Neurotrophic factors

Central nervous system neurons are post mitotic and therefore do not regenerate easily *(Beck et al 1995)*. When injured or damaged by neurotoxic substances, they undergo necrotic or apoptotic cell death *(Iwata et al 1996)*.The presence of neurotrophic factors can facilitate regeneration and the sprouting of neurites in the presence of local guidance cues *(Nguyen et al 2000)*.

Neurotrophic factors are peptides that act via retrograde signalling from target neurons as growth factors for the development and maintenance of neurons *(Yuen et al 1996)*. The development and maintenance functions include promoting neuronal survival, stimulating axonal growth and influencing axonal target finding for synaptic contacts *(Yuen et al 1996)*. Cells vie for the limited neurotrophic factors and those cells that do not obtain sufficient amounts, undergo programmed cell death *(Connor et al 1998)*. Neurotrophic factors modify neuronal dysfunction in mature neurons by modulating neuronal plasticity that emerges under degenerative conditions *(Connor et al 1998)*. Studies have also suggested that alterations in neurotrophic levels might predispose the affected areas to neurodegeneration and in some cases may even play a role in neurodegeneration characteristic of Parkinson's disease *(Connor et al 1998)*.

1.5.1 Brain derived neurotrophic factor (BDNF) and Parkinson's disease.

BDNF belongs to the nerve growth factor (NGF) super family of neurotrophic factors *(Siegel et al 2000)*. This NGF superfamily includes the neurotropin (NT) family (NT-3 to NT-6) of neurotrophic factors *(Siegel et al 2000)*. These neurotrophic factors associate as non-covalent homodimers in their active form and for neurons to be responsive to these neurotrophic factors, the transmembrane surface receptor p75 and three Tyrosine receptor kinase (Trk)

proteins; Trk A, TrkB and TrkC *(Table 1.5.1)* need to be expressed on the surface of the neurons *(Siegel et al 2000)*. BDNF has the highest affinity for TrkB while p75 can bind with low affinity to all neurotrophins *(Siegel et al 2000)*. P75 can interact with the TrK receptor family and modifies their ligand binding affinity, dose responsiveness and kinase activity *(Siegel et al 2000)*.

BDNF is a protein that has neurogenic and neuroprotective effects in the hippocampus (Alberch *et al.*, 2002, Fernandez *et al.*, 1998, Trejo *et al.*, 2001; Åberg *et al* 2000; van Praag *et al* 1999). In the presence of other neurotrophic factors and adrenal steroids, BDNF is an essential modulator of neuronal plasticity and functioning of the rat hippocampus *(Chao et al 1998)*. Adrenalectomy induces an increase in BDNF *(Schaaf et al 1998, Chao et al 1998)*. The Trk receptor family is expressed in astrocytes but not in oligodendrocytes and their expression is increased in these astrocytes (reactive astrocytes) when there is neuronal damage *(Aguado et al 1998)*.

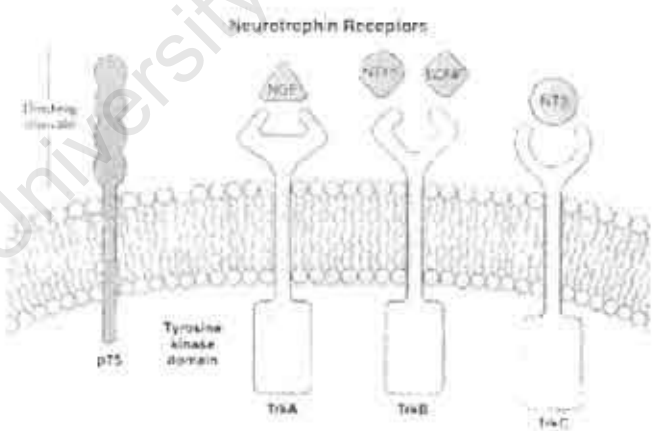


Figure 1.5.1 A representation of the binding of NGF superfamily neurotrophic factors to their preferred receptors *(Siegel et al; (2000) Brain Res Rev 33)*.

In the hippocampus BDNF is localized in the pyramidal cell layers of CA1 through CA4 and in all amygdaloid nuclei *(Murer et al 1999)*. The increase in BDNF in the hippocampus leads to an activation of hippocampal cyclic AMP response element binding protein (CREB) and synapsin-1 through calcium calmodulin kinase 11 and mitogen-activated protein kinase (MAP-K) signalling pathway *(Shen et al 2001)*. The MAP-K facilitates the phosphorylation of CREB which results in the activation of target genes that regulate structural protein, enzymes and neurotransmitters resulting in changes in neuronal function *(Shen et al 2001, Finkbeiner, 2000, Nagukura et al 2002)*. Synapsin-1 is responsible for tethering synaptic vesicles to the actin cytoskeleton and the regulation of the proportion of vesicles available for the release in the presynaptic terminal *(Jovanovic et al 2000)*.

In a Parkinsonian rat model, following lesion with 6-OHDA injected into the MFB bundle, there is an alteration of BDNF function in the striatum with increased density of TrkB receptors in the ipsilateral caudate putamen *(Numan et al 1997)*. This is thought to be a compensatory response to the loss of dopaminergic inputs or the loss of transported BDNF from the ventral midbrain *(Numan et al 1997)*. In rats, studies have shown that injecting 1-methyl-4-phenylpyridinium (MPP+), an active metabolite of MPTP into the rat striatum seven days after the implantation of BDNF-secreting fibroblasts near the substantia nigra results in neuroprotection of the dopamine neurons *(Frim et al 1994)*. Striatal injections of 6-OHDA showed evidence of increased dopamine metabolism and turnover *(Altar et al1994)*. However ELISA studies in substantia nigra and the striatum of Parkinson's diseased brains have found significantly low BDNF *(Mogi et al 1999)*. As Parkinson's disease is a progressive disease and is characterised by more than 80% dopamine neuron destruction in the substantia nigra *(Ganong et al 2005)*, the low neurotrophin levels imply that the brain can no longer prevent or delay dopamine destruction without assistance in the form of treatment.

In rats, voluntary running of 500 m per day is considered to be the threshold level needed for BDNF upregulation and thus the activation of CREB *(Shen et al 2001)*.

1.5.2 Glial cell line-derived neurotrophic factor (GDNF) and Parkinson's disease.

Glia cell line-derived neurotrophic factor (GDNF) belongs to the GDNF superfamily of neurotrophic peptides that include GDNF, neurturin, persephin and artemin all of which are structurally related to GDNF *(Siegel et al 2000)*. GDNF is the primary neurotrophic factor involved in providing support for the survival of dopamine neurons in the substantia nigra *(Kreiglestein et al 1995, Lapchak et al 1996)*. In a Parkinsonian rat model, GDNF is a potent dopaminergic trophic factor that ameliorates the behavioural and histological consequences that occur following lesion with 6-OHDA *(Connor 2000)*. GDNF has been shown to have both neuroprotective and neuroregenerative effects on dopamine neurons *(Kearns et al 1995 and 1997, Tomac et al 1995, Gash et al 1996)*. Following striatal or substantia nigra injections of 6-OHDA, GDNF has been shown to protect against the neurodegenerative effects of 6-OHDA *(Kearns et al 1995)*. When GDNF was administered after a 6-OHDA injection into the MFB, it normalized dopamine levels and increased the number of tyrosine hydroxylase immunoreactive cells in the lesioned substantia nigra *(Bowenkamp et al 1995)*. GDNF has also been shown to promote regeneration following MPTP-induced degeneration *(Tomac et al 1995, Gash et al 1996)*.

GDNF signaling involves a multi component receptor complex that includes Ret which is a member of the receptor protein tyrosine kinase (Trk) superfamily of receptors *(Trupp et al 1996)* and glycosylphosphatidyl inositol anchored protein (GPI) which is commonly called GDNF family receptor α (GFRα) *(Navailhan et al 1998)*. In the rat brain, Ret mRNA and GRFα1 mRNA are expressed in high levels in the substantia nigra *(Kokaia et al 1999)*. A physiological response to GDNF requires GRFα1 to bind to GDNF resulting in the formation of a complex

which binds to Ret thereby inducing tyrosine phosphorylation of Ret *(Siegel et al 2000)*. The activation of Ret's tyrosine kinase leads to activation of a number of intracellular downstream signaling proteins *(Siegel et al 2000)*.

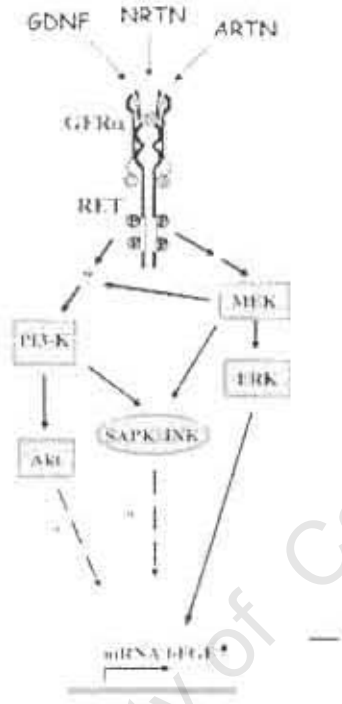


Figure 1.5.2.1 A representation of the binding of GDNF, neurturin (NRTN) and artemin (ARTN) to a receptor complex that includes GFR α and RET leading to the activation of signal proteins and gene expression *(Hauck et al; (2006) Mol Cell Biol 26 (7))*.

This network of signaling proteins has multiple interactive pathways that are necessary in cell growth *(Durik et al 1998, Porter et al 1998)*. Neurotoxic drugs such as 6-OHDA can affect the expression of GDNF receptors as was shown by studies in which the injection of 6-OHDA into the MFB resulted in a decrease in the immunoreactivity of Ret in substantia nigra and striatum *(Araujo et al 1998)*.

In rats, GDNF stimulates an increase in midbrain dopamine levels, protects dopamine neurons from some neurotoxins, and maintains injured dopamine

neurons *(Table 1.5.2, Gash et al 1996)*. Studies have shown that the injection of GDNF into the rat's striatum attenuates dopamine neuron destruction by free radicals in the nigrostriatal area of the brain following a toxic insult with 6-OHDA *(Smith et al 2007)*. Following GDNF injection into the striatum or substantia nigra of a rat, GDNF and its receptor is internalized by dopamine neurons, it starts a series of intracellular events that are based on the tyrosine kinase transduction pathway resulting in an increase in tyrosine hydroxylase positive neurons *(Ullrich et al 1990, Kearns et al 1997)*. Antioxidants such as superoxide dismutase, catalase and glutathione peroxidase also have increased activity following a single GDNF injection *(Chao et al 1999)*. As oxidative stress has been shown to be one of the major components of neurodegeneration in Parkinson's disease *(Maguire-Zeiss et al 2005)* (SECTION 1.2), GDNF plays a role in providing neuroprotection of the dopamine neurons in the substantia nigra *(Smith et al 2007)*. A signal that the tissue is undergoing oxidative damage is the appearance of protein carbonyls and 4-hydroxy-2-nonenal *(Uchida 2003)*. When tissues undergo oxidative stress, 4-hydroxy-2-nonenal attacks proteins and nucleotides causing dysfunction of the target molecules *(Uchida et al 2003)*. Increases in oxidative stress are normally detected before signs of neuronal destruction appear *(Venero et al 1997)* and therefore the neuroprotective effects of GDNF are assessed by measuring the decrease in the oxidative stress associated with 6-OHDA injection in the striatum *(Smith et al 2007)*. The increase in proteins associated with oxidative stress (protein carbonyls and 4-hydroxy-2-nonenal) is evident one day following striatal lesion with 6-OHDA but then disappears after 3 days *(Smith et al 2007b)*. However striatal injection of GDNF 3 days prior to striatal lesion with 6-OHDA has been shown to inhibit the increase in proteins associated with oxidative stress *(Smith et al 2007)*.

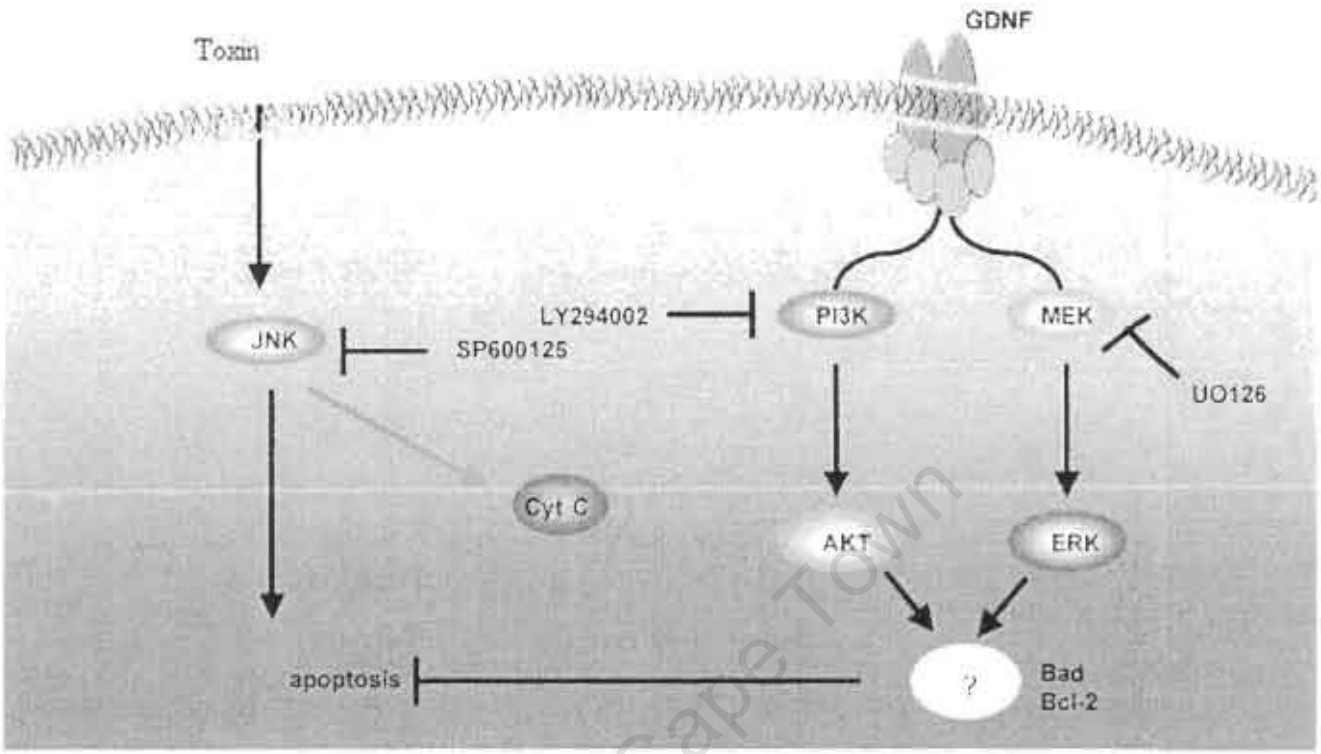


Figure 1.5.2.2 A model of the vulnerability of neurons to cell death following exposure to oxidative stress. Environmental toxins and other toxic insults such as 6-OHDA infusion result in an increase in oxidative stress and neurotrophins such as GDNF can reduce the vulnerability of the dopamine neurons to oxidative stress. *(Adapted from Villegas et al; (2006) Brain Res Bull 71 (1-3).*

In studies in which rats received unilateral 6-OHDA injection into the substantia nigra or striatum following an infusion of GDNF 24 h earlier, there was substantial neuroprotection in the striatum and substantia nigra when the rats were analysed by tyrosine hydroxylase immunohistochemistry 2 weeks later *(Kearns et al 1994)*. When GDNF was injected after MPTP lesion of the substantia nigra or striatum, dopamine levels and fibre densities were significantly restored *(Tomac et al 1995)*. Similar results were also found in studies in which GDNF was injected before MPTP lesion and in both cases, motor behaviour was increased above normal levels *(Tomac et al 1995)*.

Injection of GDNF into the striatum results in retrograde transport of this neurotrophic factor to the substantia nigra resulting in very low levels 3 days post injection *(Tomac et al 1995)*. Following injection of 6-0HDA into the striatum, a decrease in dopamine concentration was observed in the striatum only 7 days later *(Smith et al 2007)*. Activation of apoptotis is thought to initiate in the neuron terminals and terminate in the cell body *(Mattson et al 1998 and 1999)*. This has also been shown in studies where following GDNF gene transfer, reduction in dopamine neuron loss and dopamine neuron protection only occurred when gene transfer occurred in the striatum and not in the substantia nigra *(Connor 2000)*. In a Parkinsonian rat model, GDNF gene transfer leads to restrictions in transgene expression *(Don Thi et al 2006)*, and this may be one of the mechanisms with which apoptosis and thus dopamine neurons are protected. Therefore one can conclude that GDNF is one of a number of peptides present in the brain that has the ability to protect or restore neurons that have been exposed to neurotoxins such as 6-OHDA and MPTP.

1.5.3 Effects of corticosterone on expression of neurotrophic factors in the hippocampus.

In studies in which the mRNA levels of BDNF, TrkB, NT-3, and TrkC in rat hippocampal cell fields were measured after administration of different doses of corticosterone (30, 300, and 1,000 μg), the measured NT-3 and TrkC mRNA did not show significant changes in any hippocampal region after the various doses of corticosterone. However BDNF mRNA decreased after corticosterone administration dose dependently, resulting in maximal suppression of 35, 20, and 50% in the dentate gyrus, CA3, CA1, respectively *(Schaaf et al 1997)*. However TrkB mRNA responded with increased expression in the CA3 and dentate gyrus following exposure to the lowest dose of corticosterone administered (30 μg) when compared to controls but the effect of the higher doses was not different from the vehicle injected controls *(Schaaf et al 1997)*. This was thought to suggest that BDNF and TrkB expression in hippocampus is mediated by

coordinated mineralocorticoid receptor and glucocorticoid receptor function *(Schaaf et al 1997)*.

In summary, stress and thus an increase in circulating glucocorticoids is associated with decreased expression of BDNF in the dentate gyrus of the hippocampus *(Smith 1996, Chao et al 1994)*. Corticosterone administration also leads to a dose-dependent decrease in BDNF and BDNF mRNA in the hippocampus (Schaaf *et al* 1998) thus inhibiting the release of neurotrophins. If that is the case, the neuroprotective effect provided by the neurotrophins could be reduced when stressors that lead to the release of corticosterone are introduced.

1.6 Stress

Stress is defined as a state that an organism perceives as a threat to its physiological equilibrium *(Charmandari et al 2003)*. The stress response is usually of a limited duration and results in hormonal and neurotransmitter activity changes that rapidly restore to pre-stress levels when the perceived stress is no longer present *(Charmandari et al 2003)*. However when the presence of the stressor is prolonged, the organism fails to adapt to the stress resulting in the prolonged activation of the Hypothalamic-Pituitary-Adrenal axis (HPA axis) which leads to adult disorders such as hyperanxiety and depression *(Seckl 2001)*.

1.6.1 Endocrine response to stress

The response to stress involves a series of events that are mediated by the HPA axis *(Kofman 2002)*. The hypothalamic neurons involved in the HPA axis are mainly found in the parvocellular portion of the paraventricular nucleus (PVN) *(Lehnert et al 1998)*. The parvocellular portion of the PVN is divided into three parts; a medial group that produces corticotrophin releasing factor (CRF), an intermediate group that produces arginine vasopressin (AVP) and a lateral group

that produces CRF and projects to brain stem neurons *(de Goejj et al 1991)*. The PVN is the origin of pituitary afferents that control the secretion of adrenocorticotrophic hormone (ACTH) in the anterior pituitary *(Rivier et al 1986)*. Stimulation of corticotroph cells in the anterior pituitary is controlled by the synchronous pulse secretion of CRF and AVP into the portal system of the pituitary gland *(Lehnert et al 1998)*. Therefore in a stressful situation, the HPA axis response involves a sequence of neurological and endocrine changes that begin with the secretion of CRF and AVP by the parvocellular cells in the PVN *(Antoni 1993)*. The amplitude of the pulsatile secrection of CRF and AVP into the portal system increases *(Lehnert et al 1998)*, stimulating the release of ACTH from the anterior pituitary which in turn stimulates the release of glucocorticoids such as corticosterone from the adrenal gland *(Table 1.6.1, Meaney et al 1996)*.

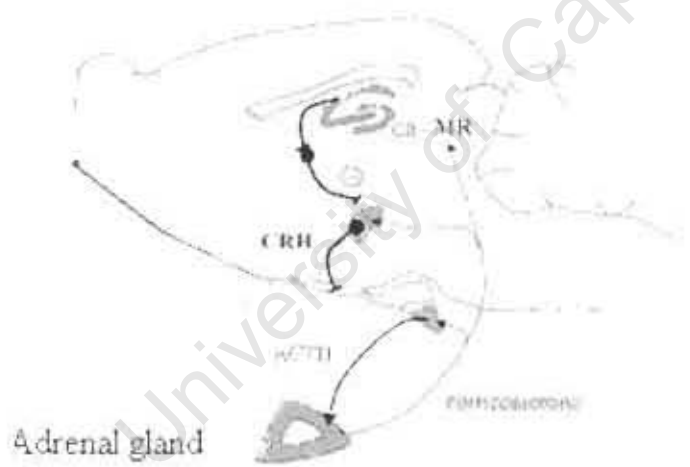


Figure1.6.1 A schematic overview of the negative feedback loop of the HPA axis with secretion of corticosterone from the adrenal cortex (ad gl) inhibiting further release of ACTH and CRH (CRF) by the pituitary gland and the hypothalamus respectively. Hippocampal mineralocorticoid receptors and glucocorticoid receptos also inhibit CRF release by the hypothalamus *(Adapted from de Kloet et al;(2004) Neurobiorev 29(2))*.

In the HPA axis cascade, CRF serves as the main regulatory protein *(Lehnert et al 1998)* by increasing pro-opiomelanocortin (POMC) mRNA transcription in the corticotrope cells of the anterior pituitary *(Antoni 1993)*.
POMC is a polypeptide that is cleaved enzymatically to produce ACTH. CRF receptor concentration in the anterior pituitary does not correlate with corticotrope cell release of ACTH. The presence of vasopressin is necessary for the pituitary to release high levels of ACTH in the presence of few CRF receptors *(Rene et al 2000, Nikodemova et al 2002)*. CRF receptors possess protein kinase C (PKC) phosphorylation sites and AVP utilizes PKC as an intracellular transducer to potentiate CRF induced ACTH secretion *(Bilezikjian et al 1987)*. The HPA axis acts via a negative feedback loop with circulating glucocorticoids acting at various sites to inhibit CRF mRNA and AVP mRNA expression *(Sapolsky et al 1984, Szuran et al 2000)*. In plasma CRF is bound to a CRF-binding protein that has a high affinity for the CRF-1 receptor thereby inhibiting the ability of CRF to stimulate ACTH release by the anterior pituitary *(Behan et al 1995)*. The negative feedback loop of the HPA axis is stimulus specific and is effective only in response to stressors that are emotional or cognitive (introduction into a novel environment) *(Plotsky et al 1993)*. Plasma glucocorticoids such as corticosterone increase under stressful conditions and are used as an index of stress reactivity *(Keenan et al 2003)*. The corticosterone response to stress along with increased catecholamine secretion, results in the suspension of anabolic functions and enhances catabolic functions that increase the availability of energy substrates such as glucose, free fatty acids and amino acids that enables the rat to adapt to or prevent the stressful condition *(Sapolsky et al 2000, Meaney et al 2000)*. Corticosteroids act on many organs via the mineralocorticoid receptors and glucocorticoid receptors *(de Kloet et al 1985)*.

In the hippocampus, high affinity mineralocorticoid receptors and low affinity glucocorticoid receptors provide a negative feedback loop to the stress response *(de Kloet et al 1998)*. The hippocampal mineralocorticoid receptors are important in the appraisal of the stress and the onset of the stress response whereas the

glucocorticoid receptors are activated by the presence of large amounts of corticosterone and are important in the termination of the stress reaction *(de Kloet et al 2004, Kofman 2002)*. The expression of glucocorticoid and mineralacorticoid receptors in the hippocamus is dependent on the availability of corticosterone with high levels of corticosterone resulting in the downregulation of the expression of these receptors *(Herman et al 1998)*. Excessive exposure to stress may lead to decreased receptor expression in the hippocampus as is the case in prenatal stress *(Kofman 2002,Szuran et al 2000, Koehl et al 1999)* leading to an abnormal HPA axis. The reduction in glucocorticoid receptor expression is also thought to lead to an attenuation of the negative feedback loop leading to elevated corticosteroid levels following stress *(Ishiwata et al 2005)*. However prolonged exposure to high levels of circulating glucocorticoids is detrimental to an organism and results in the inhibition of the formation of new cells in the hippocampus *(Gould et al 1999)*.

1.7 Prenatal stress

Prenatal stress is defined as impaired growth and development during foetal life *(Lesage et al 2002)*. Factors that are thought to cause abnormal foetal programming include maternal malnutrition and overexposure of the developing foetus to maternal glucocorticoids such as corticosterone *(Seckl 1998, Barker 2000)*.

1.7.1 Effects of nutrition during gestation.

Prenatal malnutrition in both human and animal models has been shown to have effects that last into adulthood *(Darnaudery et al 2004, Lesage et al 2002)*. Prenatal malnutrition is a serious problem causing increased stress hormone levels and decreased cognitive function in adult offspring *(deKloet et al 2003, Ward et al 2000, Griffin III et al 2003, Buitelaar et al 2003, Sternberg et al 2002)*. Maternal undernutrition has also been shown to increase blood pressure and

plasma corticosterone levels in adult offspring *(Olausson et al 2002)*. Undernutrition describes a nutritional state where all the nutritional requirements are present but in insufficient amounts whereas malnutrition refers to a state where one or more of these requirements are missing or in wrong proportions *(Morgane et al 2002)*. Protein undernutrition or malnutrition occurring during early brain formation is known to cause morphological, neurochemical and neurophysiological deficits in the developing brain *(Almeida et al 2004)*. Brain development refers to the synthesis of essential brain components such as nucleic acids and proteins that are accompanied by neurogenesis, neuronal migration and cell differentiation *(Morgane et al 2002)*. The absence or a deficiency of any of the nutritional requirements may interfere with normal brain development leading to neurological disorders. These may include hippocampal disorders such as diminished plasticity and deficits in long term potentiation *(Morgane 2002)*, altered distribution of regulatory neurotransmitters such as dopamine, serotonin, noradrenaline and acetylcholine *(Kofman, 2002)* and in some cases a reduction in weight when compared to adult age mates that were not stressed prenatally *(Almeida et al 2004)*. Altered reduction of dopamine includes reduced dopamine turnover in the striatum and nucleus accumbens *(Kofman 2002)* in the brains of rats that were affected by mild or severe prenatal stress thus developing a mild prenatal stress model might be important in understanding the role early life stressors such as prenatal stress play in the development of neurodegenerative diseases such as Parkinson's disease.

1.7.2 Hypothalamo-Pituitary-Adrenal Axis development during gestation.

During the first two weeks of gestation, the neonatal HPA axis is nonresponsive to stress but during the last week of gestation the foetal rats respond to stress with increased corticosterone levels (Kofman, 2002, Dallman, 2000). The timing of maturation of the HPA axis relative to birth is highly species specific, and is closely linked to landmarks of brain development *(Dobbing et al 1979)*. In rats glucocorticoid receptors and mineralocorticoid receptors are expressed at low

levels in the brain throughout gestation with glucocorticoid receptor mRNA present in the hippocampus, hypothalamus and pituitary by gestational day 13 while mineralocorticoid receptor expression is not present in the hippocampus until gestational days 16–17 *(Matthews 2002)*. Glucocorticoid receptor mRNA starts increasing towards the end of term (E21) and both glucocorticoid and mineralocorticoid receptor expression rapidly increase after birth which is consistent with the postnatal nature of development of the brain and HPA axis development in rats *(Matthews 2002)*. The prenatal development of the CRH system also coincides with the last week of gestation with CRH receptors and CRH mRNA that are crucial for the regulation of the HPA axis detectible by gestational days 16 and 17 *(Eghbal-Ahmadi et al 1998, Griffin et al 2003)*.

1.7.3 11ß-hydroxysteroid dehydrogenase (11ß-HSB)

Stress during gestation results in the secretion of an effector molecule such as corticosterone which is lipophilic and can pass through the foeto-placental barrier *(Griffin 111 et al 2003)*. In the third week of gestation, maternal corticosterone levels are higher than in the first two weeks *(Atkinson et al 1995)*. The developing foetus is protected from relatively high levels of maternal corticosteroids by the enzyme 11ß-hydroxysteroid dehydrogenase2 (11ß-HSB2) which breaks down active corticosterone to the inert molecule 11-dehydrocorticosterone *(Siebe et al 1993, Griffin 111 et al 2003)*. The enzyme11ß-HSB 2 is highly expressed in the labyrinth zone of the placenta and in other foetal brain tissue such as the cerebellum, thallumus, pons and hippocampus *(Brown et al 1996)*. This enzyme is most active during gestational day 16 after which its activity decreases *(Waddell et al 1998)*. Despite11ß-HSB 2's high affinity for corticosterone, it does not completely block its transmission from maternal circulation to foetal circulation *(Brown et al 1993)*. Studies have shown that approximately 80% of glucocorticoids are metabolized by the enzyme11ß-HSB 2 in the human placenta *(Benediktsson 1997)*. In rats, the activity of 11ß-HSB 2 has individual variation and correlates positively with birth weight *(Benediktsson 1993)*. Rats that had low

placental 11ß-HSB 2 activity had low birth weight *(Benediktsson 1993)*. It is the exposure of the foetus to these high maternal corticosterone levels that results in neurobiological changes in the developing brain *(Weinstock et al 1997)*. These neurobiological changes manifest themselves as abnormal behavioural characteristics in tests performed on adult offspring (*Griffin 111et al 2003)*. Therefore for an acute prenatal stress paradigm, the pregnant dams should be stressed in the last week of gestation when the maternal plasma corticosterone levels are elevated and the placental 11ß-HSB 2 activity is diminished. This has been supported by studies which showed that when the rats were stressed in the last week of gestation (E14-19), when the activity of 11ß-HSB 2 is low, there was increased corticosterone in foetal plasma which was still elevated two days after the termination of the stress *(Takahashi et al 1998)*. Also when 11ß-HSB 2 was inhibited by glycyrrhetinic acid, cortisol was able to cross into the foetal circulation *(Benediktsson 1997)*. Therefore foetal overexposure to maternal corticosteroids due to prenatal stress or reduced activity of foeto-placental 11ß-HSB 2 may represent a link between the prenatal environment, foetal growth and adult neuroendocrine and affective disorders.

1.7.4 Long lasting effects of prenatal stress on the developing brain.

Stressing the rats during the last week of gestation can interfere with the development of neural networks *(Sternberg et al 2003)* that control physical and motor development, exploration in a novel environment and behavior under stressful conditions *(Buitelaar et al 2003)*. These effects are thought to have been caused by a disturbance in the development of the HPA axis as a result of overexposure of the foetus to maternal corticosterone (Lesage *et al*., 2002). The mechanism, with which this disturbance occurs, is thought to involve Fos protein expression as studies have shown that in adult offspring of prenatally-stressed rats, there were no changs in Fos immunoreactivity in the hippocampus before or after an acute stressor *(Viltart et al 2006)*. This is thought to suggest an altered neuronal activation in the hippocampus and thus the HPA axis in adult offspring

of prenatally-stressed rats *(Viltart et al 2006)*. The stress response works via a negative feedback system that utilizes mineralocorticoid and glucocorticoid receptors in the hippocampus *(Figure 1.6.1, Szuran et al 2000)*. Prenatal stress has been shown to result in a decrease in glucocorticoid receptors in the hippocampus which leads to disruption of the negative feedback loop resulting and an increase in ACTH and corticosterone levels (Ishiwata *et al.*, 2005). The formation of the neural circuitry that controls HPA axis function is said to continue into the first weeks post partum (Sternberg *et al* 2003). Studies have shown that the foetal HPA axis is functional during late gestation; it is then suppressed in the first two weeks post partum and starts to operate at adult levels in the third week post partum (Kofman., 2002; Meaney *et al.*, 1986). The glucocorticoid receptor concentration in the hippocampus is also low during the first 3 weeks post partum (Ishiwata *et al.*, 2005).Therefore stressing the rats during the perinatal period might interfere with the formation of this circuitry.

Studies have shown that by creating a prenatal stress rat model, it is possible to mimic these developmental and behavioural alterations *(Darnaudery et al 2004, Lesage et al 2002, Ward et al 2000)*. These rats show a reduced propensity for social interaction, increased anxiety in intimidating or novel situations and a reduction in cerebral asymmetry and dopamine turnover which results in behavioural abnormalities commonly associated with impaired regulation of the HPA axis response to stress and increased CRH activity *(Weinstock 2001)*. It is the increase in maternal corticosterone levels that is the programming factor in the prenatal stress paradigm *(Muneoka et al 1997)* by causing high corticosterone levels in foetal circulation to alter the HPA axis of the pups exposed to prenatal stress *(Zagron et al 2006)*. In adult offspring, prenatal stress impairs the negative feedback loop of the HPA axis by decreasing the hippocampal glucocorticoid and mineralocorticoid receptors *(Henry et al 1994)* as a result exposing rats that were prenatally stressed to an acute stressor, can start a series of events that lead to an elevated corticosterone in plasma and the central nervous system *(Castro et al 2001)*. Adult prenatally stressed rats have a

hyperactive HPA axis and this is indicated by a higher or prolonged stress hormone response to stress *(Griffin 111et al 2003)*.

1.7.5 Prenatal stress models

Models of gestational stress that have been used include food deprivation models (Kehoe *et al* 2001, Lesage *et al* 2002, Ježová *et al* 2002) henceforth refered to as the Kehoe, Lesage and Ježová models, the pregnant rats were food deprived during gestation. Other prenatal stress models included the mild stressor model in which the rats were subjected to variable stressors including daily handling and saline injections during the last week of gestation (Ward *et al* 2000), the rodent stress model in which the rats were subjected to restraint stress at different time points during gestation *(Fujioka et al 2001, Ishiwata et al 2005)* and prenatal stress that is caused by the injection of 11ß-HSB2 inhibitors during gestation *(Benediktsson et al 1997, Welberg et al 2000)*. The food deprived models can be divided into undernutrion *(Kehoe et al 2001)* and malnutriotion *(Ježová et al 2002, Lesage et al 2002)* models. In the Kehoe model the protein content was reduced to 6% casein (normal= 25% casein) and was therefore a form of prenatal undernutrion (SECTION 1.4.1.2). Offspring of rats that were undernourished from 5 weeks prior to mating up until partuition had a lower corticosterone response to an acute or chronic isolation stress *(Kehoe et al 2001)*. In the malnourished groups (SECTION 1.4.1.2), food deprivation prenatal stress can be further subdivided to acute and chronic. In the acute prenatal stress model proposed by Ježová, food deprivation occurred in the last week of gestation (E14-21) whereas in the chronic model suggested by Lesage, food deprivation continued from E 14 until weaning on P 21. In both the acute and chronic models, rats received 50% of the food consumed by the control rats *(Lesage et al 2002, Ježová et al 2002)*. In the Ježová model, there was an increase in circulating ACTH concentration in adult offspring but no change in the corticosterone levels whereas in the Lesage model, the adult offspring had elevated corticosterone levels but a blunted ACTH response following restraint

and the corticosterone levels returned to basal levels more quickly than in the control rats *(Lesage et al 2002)*.

Prenatal restraint protocols can be classified as either severe or mild. In the mild model of prenatal stress due to restraint, the rats were restrained daily for a period of 30 min from E15-17 *(Fujioka et al 2001)*. There was no difference in corticosterone levels of the adult offspring of rats that were prenatally stressed and control rats following physical and psychological stress however the prenatally stressed rats exhibited anxiety-like behaviour when exposed to an open field apparatus *(Fujioka et al 2001)*. Anxiety-like behaviour in the open field is described as reduced locomotor activity when compared to controls *(McFadyen-Leussis et al 2004)*. In severe forms of prenatal restraint stress, the rats were subjected to 45 min restraint in a rodent holder three times a day from E15-21 *(Ishiwata et al 2005)*. There was no difference in the basal corticosterone levels of the prenatally-stressed and control rats but following restraint, the corticosterone levels of the rats that were subjected to the prenatal stress protocol were significantly elevated *(Ishiwata et al 2005)*. In studies in which the pregnant dams were injected with carbenoxolone which blocks the activity of the enzyme11ß-HSB2 from the first day of gestation until partuition, adult offspring had elevated basal corticosterone levels and CRH but decreased glucocorticoid receptor mRNA in the PVN of the hypothalamus and normal glucocorticoid receptor mRNA and mineralocorticoid receptor mRNA in the hippocampus *(Welberg et al 2000)*.

These prenatal stressors have different effects on the behavior of adult offspring in a novel environment and stress response following exposure to an acute stressor. The differences in the response to these parameters might be due to the severity or duration of the prenatal stressor used.

1.8 Maternal separation

Traumatic early life experiences have long lasting effects on the HPA axis and are believed to play a major role in the development of adult anxiety disorders *(Heim et al 2001)*. In rats early life stressors such as maternal separation in the first weeks after birth can permanently alter the HPA axis (stress response) *(Meaney et al 1989)* leading to increased basal CRF mRNA in the hypothalamic and extra hypothalamic nuclei in adult rats *(Hout et al 2002)* and adverse effects on the behaviour of rats in adulthood *(Daniels et al 2004)*.

1.8.1 The Stress-Hypo-Responsive Period

Immediately after birth, pups have high circulating corticosterone levels which gradually decline on postnatal day (P) 1 and 2 and remain low during the stress-hyporesponsive period *(Walker et al 1986)*. The stress-hyporesponsive period is crucial in protecting the developing rats from the effects of mild stressors as the synthesis of CRF and ACTH by the PVN of the hypothalumus and the anterior pituitary respectively are reduced *(Walker et al 1986 and Sapolsky et al 1986)*. The stress-hyporesponsive period begins on P 4 and lasts until P 14 *(Hout et al 2002)* and is characterised by low circulating corticosterone levels and the inability of mild stressors to induce a corticosterone response *(Figure 1.8.1, Levine et al 1992)*. The low levels of corticosterone are due to the adrenal glands' insensitivity to the low levels of circulating ACTH *(Schmidt et al 2004)*. The glucocorticoid receptor concentration in the hippocampus is also low during the first 3 weeks post partum *(Ishiwata et al 2005)*. A single maternal separation period of less than 2 h is not sufficient to trigger a corticosterone response as a result of adrenal insensitivity to the circulating ACTH due to the stress-hyporesponsive period *(Levine et al 1991)*.

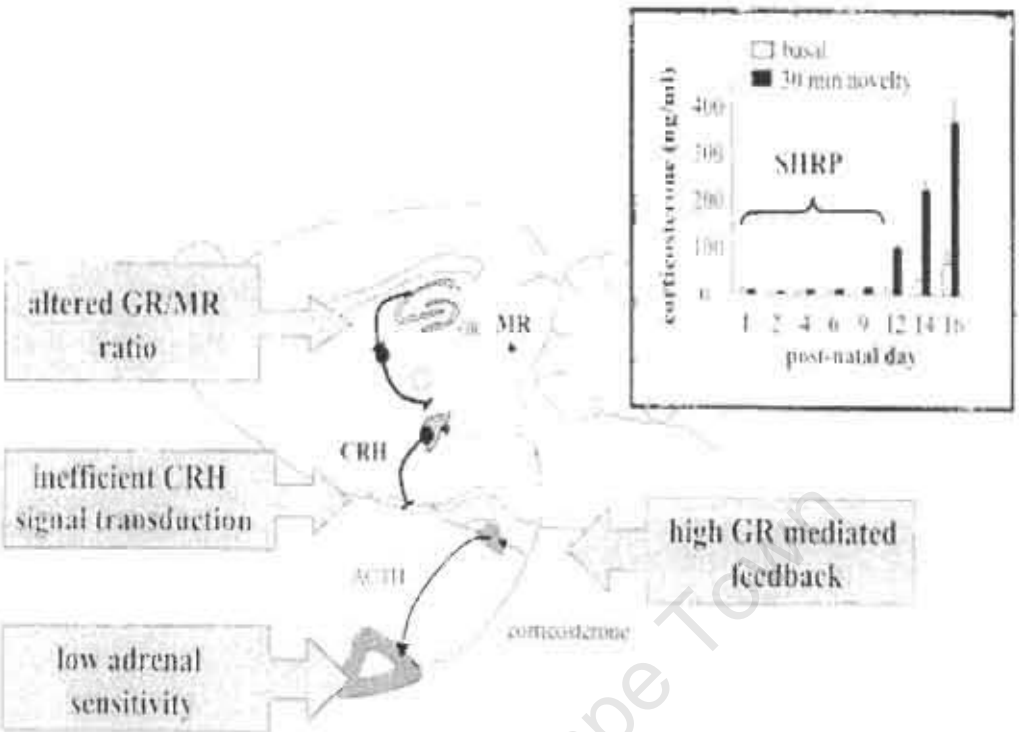


Figure 1.8.1 A schematic overview of the negative feedback loop of the HPA axis during the stress-hyporesponsive period. The HPA axis activity is decreased by adrenal insensitivity to ACTH and the inhibitory effect of corticosterone on POMC via glucorticoid receptors in the hippocampus. Mineralocorticoid receptor (MR) *(de Kloet et al; (2004) Neurobiorev 29(2))*.

However separating the pups from the dam for twenty-four hours has been shown to lead to an increase in basal and stress induced corticosterone levels on PND 9 *(Schmidt et al 2002)* as the stress of maternal separation is able to disinhibit the stress-hyporesponsive period blockade of the HPA axis *(Schmidt et al 2006)* by increasing CRF mRNA synthesis in the hypothalamus *(Plotsky et al 1993)*.

1.8.2 Effects of Maternal separation in the first two weeks after birth

In maternal separation an increase in ACTH and corticosterone release occurs when the pups are separated from the dam for a period of more than 2 h and corticosterone levels reach maximal levels 12 h after separation *(Schmidt et al 2004)*. Adrenal insensitivity to the circulating ACTH is overcome when the maternal separation exceeds 8 h *(Schmidt et al 2004)*.

The activation of the HPA axis during this period (24 h separation) is thought to be due to the absence of maternal behaviour such as licking and feeding the pups which inhibits the HPA axis of the pups *(van Oers et al 1998, Lau et al 2004)*. However maternal separation models of less than 2 h can achieve the same adrenal sensitivity to the circulating ACTH as longer duration models (24h) if the separation paradigm is repeated over a number of days (PND1-8) *(McCormick et al 1998)*.

1.8.3 Maternal separation models

Maternal separation (as a stressor) is used as a model to study long-term neurochemical and behavioural changes in adult rats *(Daniels et al 2003, McCormick et al 2002)*. These studies looked at behavioural and hormonal differences in adult rats that were maternally separated in the first two weeks post partum when compared to controls *(Daniels et al 2003, Meaney et al 1989)*. Maternal separation paradigms may either consist of a repeated short-term separation (3 h per day) from P 2-14 *(Meaney et al 1989)* or a single long-term separation (24 h) *(Lehmann et al 1999, Barna et al 2003)*. However there has been a discrepancy in the behavioural analysis of anxiety in adult rats with some studies using the short-term separation protocol *(McIntosch et al 1999)* or the long-term protocol *(Lehmann et al 1999)* showing a lack of anxiety-like behaviour while in other studies rats that underwent the short-term protocol *(Kalinichev et al*

2002) or the long-term protocol *(Penske et al 2001)* were more anxious in the elevated plus maze.

The effects of maternal separation on the neuroendocrine system may not be inherent but may be exacerbated by a reaction to an acute stressor *(Sapolsky et al 1986)*. Studies have shown that glucocorticoid levels only differed from non-stressed rats after exposure to an acute stress in both the short-term protocol *(Kalinichev et al 2002)* and the long-term protocol *(Lehmann et al 2002)*. In the striatum, following an acute restraint stress in adult mice, there was an increase in the dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytryamine (3-MT) in rats that were exposed to the repeated short-term maternal separation paradigm in the first two weeks post partum *(Cabib et al 1993)*. This suggests that the neural response to acute stressor in maternally separated rats is not limited to the HPA axis.

1.9 CONCLUSION

Studies have shown that creating a Parkinsonian rat model by injecting 6-OHDA into the MFB is well established and reproducible. Exercise has been shown to have beneficial effects in alleviating the motor function asymmetry that develops as a result of unilateral 6-OHDA lesions in the nigrostriatal pathway of rats. One method by which exercise alleviates motor function asymmetry in unilateral 6-OHDA lesioned rats is thought to be by protecting the neurons from the toxic effects of 6-OHDA. Neuroprotection occurs as a result of an increase in neurotrophic factors such as BDNF and GDNF which are sensitive to an increase in circulating corticosterone a key hormone in the regulation of the HPA axis. Plasma corticosterone levels can be manipulated by the addition of stressors that may result in acute or chronic elevation in circulating corticosterone. These stressors may be applied to pregnant dams or to pups in the first two weeks after birth resulting in prenatal and maternally separation stress models respectively. Other than affecting neurotrophic factor expression, increased corticosterone

levels have also been associated with abnormal CNS formation which results in the brain being more susceptible to injury later on in life.

Most studies that looked at the neuroprotective effects of exercise following 6-OHDA lesions have focused on using treadmill running or forced use of the impaired limb. These exercise protocols fall under the category of forced exercise and may not be practical as therapy in severely affected Parkinson's disease patients. Forced exercise may also have the unintended consequence of being stressful and thus increasing circulating corticosterone. The neuroprotective effects of GDNF have mainly been investigated in non-exercise models of GDNF expression such as GDNF gene transfer or by injecting GDNF directly into the relevant regions of the basal ganglia.

The aim of looking at therapeutic modalities in Parkinsonian rat models is so that they can be used in the treatment of Parkinson's disease patients. This means that wherever possible the interventions used should be practical to a clinical setting. Our hypothesis is that more practical forms of exercise such as voluntary running that can be mimicked by Parkinson's disease patients may provide neuroprotection in exercising rats. Therefore we aim to investigate whether;

(i) voluntary exercise provides neuroprotection in 6-OHDA lesioned rats.

(ii) exposure to stress affects GDNF expression and thus its neuroprotective effects in the nigrostriatal pathway of 6-OHDA lesioned rats.

(iii) an uninterrupted 3 week exposure to voluntary wheel running affects the HPA axis and GDNF expression in non-lesioned rats

We plan to create a mild prenatal stress model that can be used to look at whether;

(i) brain injury is exacerbated by the infusion of a small dose of 6-OHDA and (ii) whether voluntary exercise can reverse the susceptibility of the brain to the neurotoxic effects of 6-OHDA.

We also plan to investigate whether the vulnerability of the brain to injury in maternal separation is of equal severity to that seen in prenatally stressed rats.

CHAPTER 2

Voluntary Running Provides Neuroprotection in Rats after 6-Hydroxydopamine Injection into the Medial Forebrain Bundle.

(Part of this work was published in *Mabandla et al; (2004) Met Brain Dis (19), 43-50).*

2.1 INTRODUCTION

Studying the pathophysiology of Parkinson's disease relies on experimental animal models *(Blandini et al 2007, Section 1.3).* 6-OHDA-induced lesions of the nigrostriatal pathway in rats remain the most widely used mode of developing a Parkinsonian rat model due to their reproducibility and the low complexity of the procedure *(Blandini et al 2007).* When 6-OHDA is infused into the nigrostriatal pathway, it rapidly undergoes auto-oxidation in the extracellular space resulting in the formation of reactive oxygen species which cause dopamine neuron cell death *(Hanrott et al 2006, Section 1.3).* Following unilateral 6-OHDA infusion, subsequent injections of dopamine agonists induce rotational behaviour in rats *(Ungerstedt 1971, 1976).* The dopamine agonist apomorphine which produces contralateral rotations following unilateral 6-OHDA infusion is a reliable measure for the determination of the extent of dopamine neuron destruction *(Ungerstedt 1971, 1976).*

Exercise has been shown to increase the availability of intra- and extra-brain neurotrophic peptides following injury in different areas of the brain *(Section 1.4).* These neurotrophic peptides have been shown to have both neurogenic and neuroprotective effects in the affected brain regions *(Section 1.4, 1.5.1, 1.5.2).*

Neurodegenerative diseases such as Parkinson's disease are characterised by a progressive loss of more than 80% of the dopaminergic neurons in the substantia nigra *(Section 1.2).* If neurogenesis and/or neuroprotection could be promoted in the substantia nigra of persons suffering from Parkinson's

disease by an exercise regimen it could prove to be very beneficial in attenuating the disease progress. In studies in which rats were forced to exercise the impaired limb following unilateral 6-OHDA injection into the medial forebrain bundle, it was found that there was complete sparing of the dopamine neurons in the substantia nigra on the ipsilateral side (Tillerson *et al 2001).* However in this study there appears to be a critical exercise period since if there was a delay in using the impaired limb (3 or 7 days) there was partial sparing and complete loss of the dopamine neurons respectively.

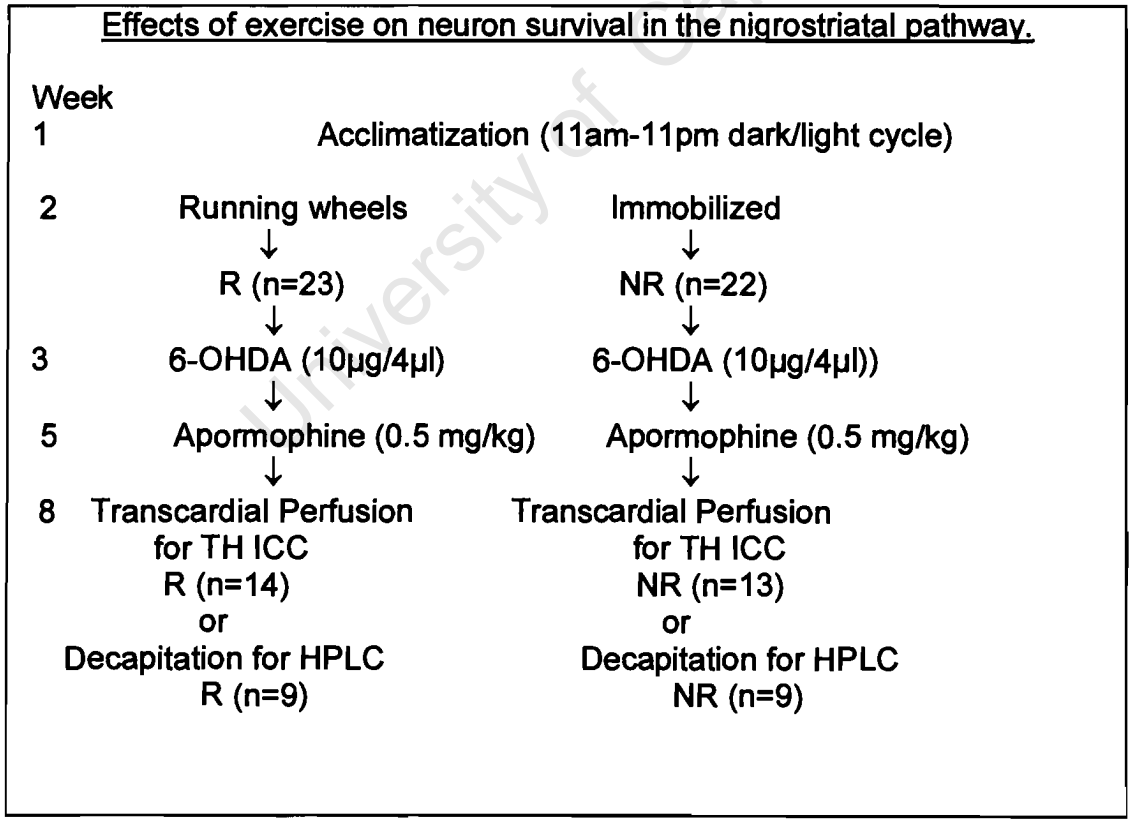
The common factor in these studies is that the rats were forced to exercise either by casting the unimpaired limb or by treadmill running. One of the symptoms of clinical Parkinson's disease is the paucity of movements *(Section 1.2)* which would make compliance to stressful exercise regimens unlikely in Parkinson's disease patients *(O'Dell et al 2007).*

This then raises the question of whether voluntary exercise can cause neuroprotection of dopamine neurons in the substantia nigra. The aim of this study was therefore to investigate if voluntary exercise can provide neuroprotection to the dopamine neurons in substantia nigra/striatal area after a neurotoxic insult.

2.2 MATERIALS and METHODS

Forty five male Long Evans rats (250-280 g) were collected from the Faculty of Health Sciences animal unit, weighed and then housed in plexiglass cages in the departmental animal house experimental room for a minimum of 7 days to acclimatise to a 12 hr (11pm to 11am) inverted light-dark cycle *(Table 2.2)*. Rats were allowed free access to commercial pellet food and tap water.

Table 2.2 Flow diagram of experimental protocol. Lesioned (R) rats with running wheels attached and lesioned (NR) rats with immobilized running wheels were either transcardially perfused for tyrosine hydroxylase (TH) Immunohistochemistry (ICC) or decapitated for striatal dopamine measurements by HPLC.



2.2.1 Running experiments

After 7 days, the rats were weighed and divided in to two groups. Eleven rats (experimental) were put into cages that had free running wheels attached to them and 10 rats (control) were put into cages that had their running wheels immobilised. The revolutions of the free running wheels were recorded daily between 09:30 and 10:30 i.e. between 30 minutes and 90 minutes before the commencement of the dark cycle at 11 am. The rats in the experimental group were free to use the wheels *ad libitum*. In the third week of the experiment, the rats were weighed and then taken to the surgical laboratory to prepare for stereotaxic surgery. The rats were taken to the surgical laboratory at least an hour before stereotaxic surgery began.

2.2.2 Stereotaxic surgery.

The rats weighed between 280 and 320 grams at the time of stereotaxic surgery. The rats were anaesthetised using a mixture of oxygen and halothane administered via a calibrated Blease Vaporiser (DATUM). After exposing the skull by making a midline incision with a scalpel, a burr hole was constructed above the target area (see coordinates below). Both experimental and control rats received 6-OHDA HCL (10 µg/4 µl saline; Sigma, St. Louis,MO, U.S.A) infusion unilaterally (0.5 µl/min) using a 32G dental needle into the left medial forebrain bundle (4.7 mm anterior to lambda, 1.6 mm lateral to midline and 8.4 mm ventral to dura, Paxinos *et al* 1986, coordinates, *Guan et al 2000*). After surgery the rats were allowed to recover in plexiglass cages for two hours before they were returned to their respective cages. The number of revolutions of the free running wheels was recorded daily for a period of two weeks post surgery. The daily recordings were taken at the same time as the pre surgery recordings.
Two weeks post surgery (week 5 of experiment) the rats were taken out of their cages and weighed. The rats were placed in plexiglass cages in readiness for apomorphine-induced rotations.

2.2.3 Apomorphine-induced rotation.

After weighing, the rats were placed individually in a plastic drum-rotometer at 11am (commencement of dark cycle, first four rats) for a period of 30 minutes. They were then injected with the dopamine receptor agonist apomorphine (0.5 mg/kg, s.c., Sigma, St. Louis,MO, U.S.A) and their rotations were recorded for a further 90 minutes. If more than four rats were being tested on one day, then the subsequent group of rats were placed into the drums immediately after completion of the first group i.e. at 1pm, two hours into the dark cycle. The net rotations that each rat made could be ipsilateral or contralareral to the site of 6-OHDA injection. Net rotation = (contralateral rotations – ipsilateral rotations).

2.2.4 Transcardial Perfusion

The rats were deeply anaesthetized in an airtight container containing 3.5% halothane oxygen mixture pumped from a Bleaze vapouriser (DATUM). After ascertaining that the rat was unconscious, it was placed on its back on the dissection tray. Using a pair of sharp scissors, a midline surgical cut along the chest was made to expose the rib cage and diaphragm. A midline cut through the diaphragm was made to expose the heart. The pericardium was exposed by making lateral cuts bissecting the ribs and then reflecting the ribs. An 18 gauge needle was inserted into the left ventricle and using gravity feed, the stopcock was opened and the rat perfused with 0.15M Phosphate Buffered Saline (PBS) solution (MERCK, Germany) which was suspended above the animal. Using a pair of sharp forceps, the right atrium was punctured to allow the blood, PBS and fixative (to follow) to leave the body during the perfusion. The abdominal aorta was clamped and after perfusing the rat with 150 ml PBS, the fixative stopcock was opened (closing the PBS stopcock) and the rat was perfused with 300 ml of 4% parafomaldehyde (PFA) (MERCK, Germany) or until the animal became rigid. Using bone cutters, the skull was removed and the brain scooped from the calverium. The brains were post fixed in 4% paraformaldehyde for 24 hrs and

then cryoprotected in 20% sucrose for 48 hrs after which they were stored in a -80°C freezer until needed for tyrosine hydroxylase immunohistochemistry.

2.2.5 Cryostat cutting

Sixty micron (µm) sections were cut antero-posterially until the striatum was visible. A metal prong (1 mm in diameter) was driven through the striatum to the back of the right hemisphere (non-lesioned hemisphere). The hole made was used as a marker to differentiate the two hemispheres after immunohistochemistry. The brain was sliced coronally and striatal and substantia nigra tissue was collected in the -20 °C environment of the cryostat machine.

2.2.6 Immunohistochemistry

The slices were washed in PBS (0.15M, pH 7.6) and then incubated for 15 min in 3% hydrogen peroxide to quench endogenous peroxidase activity. Following quenching, the slices were washed several times in PBS and then incubated for 1 h in blocking solution containing PBS, normal horse serum (Vectastain) and Triton-X. Following the blocking step, the slices were incubated in primary monoclonal anti-Tyrosine hydroxylase mouse antibody (Vectastain) at 1:16000 dilution. The slices were kept in a 4°C fridge for 2 days. The slices were washed in PBS and then incubated in biotinylated secondary antibody (Vectastain) for 90 min to 2 h. After washing with PBS, the slices were incubated for 90 min in Vectastain PK-6102, Mouse IgG ABC reagent (Vectastain) prepared 90 min before use. The slices were washed and then pretreated in diaminobenzidine tetrahydrochloride tablets (DAB) in Tris buffer (pH 7.2, Sigma) at room temperature for 10 min or until staining appeared. The slices were washed 6 times with distilled water and then mounted onto gelatinized glass slides (10 g commercial gelatine in 500ml distilled water).

2.2.7 Mounting

The slides were placed in glass slide holders, dehydrated in increasing concentrations of ethanol (96-100%) for 1 min in each concentration. The slides were cleared in xylol for 2 min and then cover slips were placed over the slices using Entellan (MERCK, Germany).

2.2.8 Counting

The tyrosine hydroxylase positive dopamine neurons in the substantia nigra of both hemispheres were counted using a Nikon Microphot-fx microscope (10x magnification). Only complete dopamine neurons with stained cell bodies, dendrites and axons were counted.

2.2.9 High Performance Liquid Chromatography (HPLC)

Nine rats from each group were sacrificed by decapitation. Following decapitation, the skull was removed with the aid of bone cutters. The brain was scooped from the calverium and cooled in ice cold 0.9% saline for at least 5 min so as to slow down protease enzyme activity. A 2.0 mm coronal section (anterior/posterior: 0.0–2.0 mm to bregma) was then removed using an ice-cold scalpel. Both striata were dissected out on ice, weighed, flash frozen in liquid nitrogen, and then stored in liquid nitrogen until removed for striatal dopamine and DOPAC quantification by high performance liquid chromatography (HPLC).

2.2.9.1 Sample preparation.

The samples were removed from the liquid nitrogen container and diluted 20 times their wet weight with 0.1 M perchloric acid (20 ul for each mg of tissue). which should be added to the tube containing the tissue. The tissue was homogenised by sonication and then centrifuged at 18000 rpm for 30 min at 4°C. The supernatant was collected and stored in a -80°C freezer.

2.2.9.2 HPLC analysis

For the analysis of dopamine and its metabolite DOPAC, a Waters 1525 HPLC pump (Waters Corp., Milford, MA, USA) was used. The mobile phase consisted of 17.5% acetonitrile, 82.5% citric buffer with 2mM decane-sulfonic acid sodium salt in HPLC grade water, pH 3.5. A Waters Symmetry C18 4.6 × 150 mm column with a 5 µm particle size was used for separation. A Waters 2465 electrochemical detector set at 750 mV relative to a salt-bridge Ag/AgCl reference electrode was used for dopamine and DOPAC detection. The flow rate was 1.0 ml/min and dopamine and DOPAC concentrations were determined by the generation of a calibration curve using peak height of external standards for dopamine and DOPAC (both from Sigma-Aldrich Corporation; St. Louis, MO, USA).

2.3 STATISTICAL ANALYSIS.

Graph Pad Prism 4 was used for statistical analysis of the HPLC results. ANOVA was used to analyse the data and when significant differences were found ($p<0.05$), post hoc comparison using Tukey's Multiple Comparison Test were performed. Where there were no multiple comparisons (Tyrosine hydroxylase Immunohistochemistry and percent dopamine destruction in the striatum), non-parametric unpaired t tests were used to analyse the data. All results are reported as mean ± standard error of the mean (SEM).

Table 2.5.4.4 Ratio of dopamine to DOPAC in the lesioned striata of rats (R) with running wheels attached and rats (NR) in cages with immobilised wheels.* (NR lesioned vs NR non lesioned, $p<0.05$)

Dopamine to DOPAC ratio			
Lesioned		Non-Lesioned	
R	NR	R	NR
3.3 ± 0.12	3.6 ± 1.08	4.2 ± 1.02	6.1 ± 1.11*

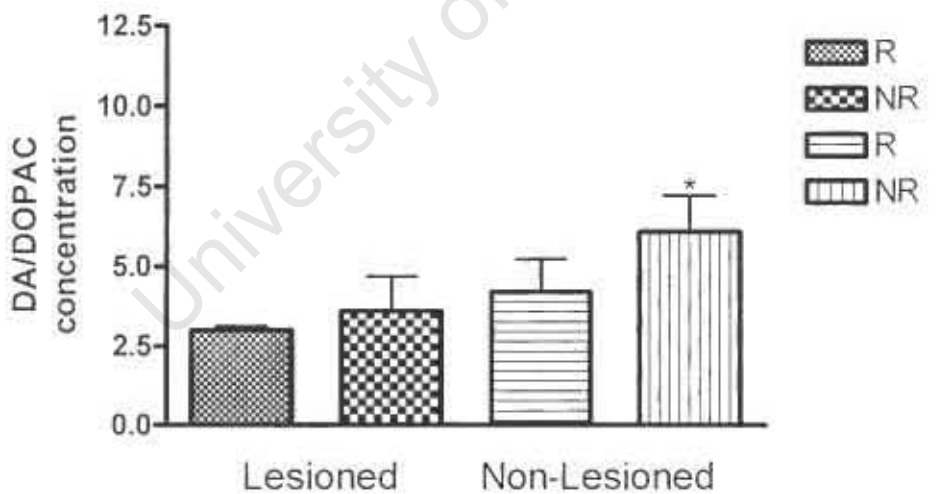


Figure 2.5.4.3 Ratio of dopamine to DOPAC in the lesioned striata of rats (R, n=9) with access to running wheels attached and rats (NR, n=9) in cages with immobilised wheels. .* (NR lesioned vs NR non lesioned, $p<0.05$)

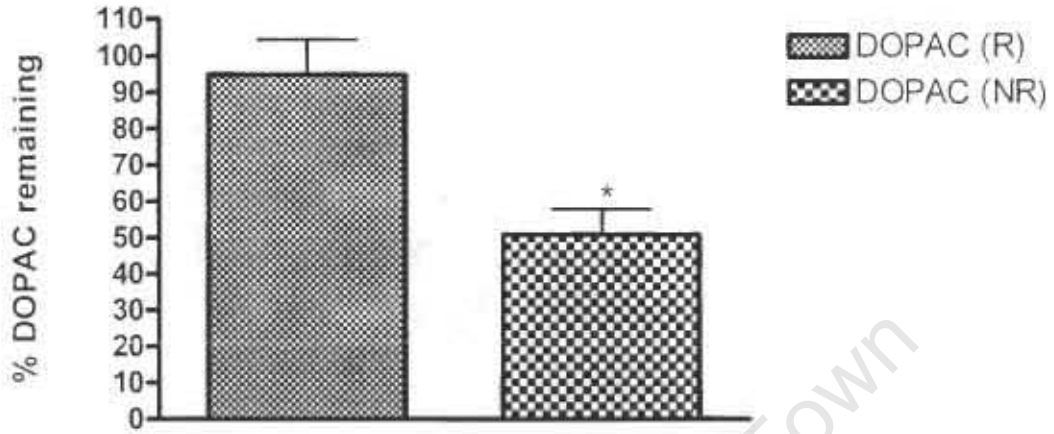


Figure 2.4.5.3.1 Percentage DOPAC the lesioned striatum of rats that had access to running wheels (R, n=9) and the percentage DOPAC concentration in the lesioned striatum of rats in cages with immobilised running wheels (NR, n=9). *(R vs NR, $p<0.002$). Data reported in Table 2.4.5.3.

2.4.5.4 Dopamine to DOPAC ratio

There was no significant difference between the dopamine to DOPAC ratio in the striata of rats (R) in cages with running wheels. There was a significant difference between the dopamine to DOPAC ratio in the striata of rats in cages without running wheels *(Table 2.4.5.4, Figure 2.4.5.4)*.

Table 2.4.5.3 The percentage of striatal DOPAC remaining in the lesioned hemisphere of rats with running wheels attached and the percent of striatal DOPAC remaining in the lesioned hemisphere of rats in cages with immobilised wheels (NR). *(R vs NR p<0.002).

DOPAC concentration (ng/g wet weight).

Non-lesion(R)	Lesion(R)	Non-lesion(NR)	Lesion (NR)
466 ± 89.0	442 ± 85.1	634 ± 103	323 ± 72.7

% DOPAC remaining in the lesioned hemisphere.

R	NR
94.8 ± 9.56	50.9 ± 7.09*

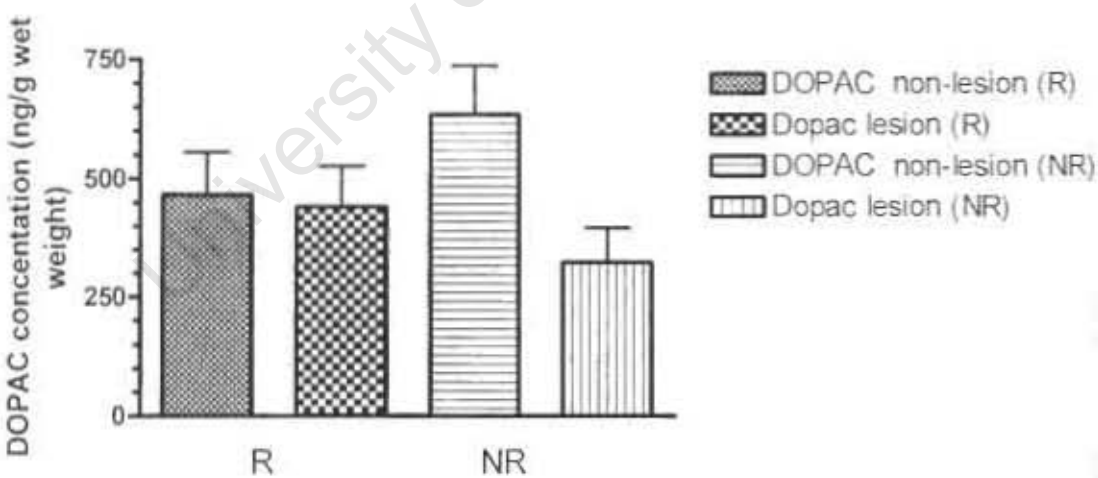


Figure 2.4.5.3 DOPAC concentration in the striatum of rats that had access to free running wheels (R, n=9) and rats that had their running wheels immobilised (NR, n=9). DOPAC concentration was measured in the non-lesioned and lesioned hemispheres of the rats. Data reported in Table 2.4.5.3.

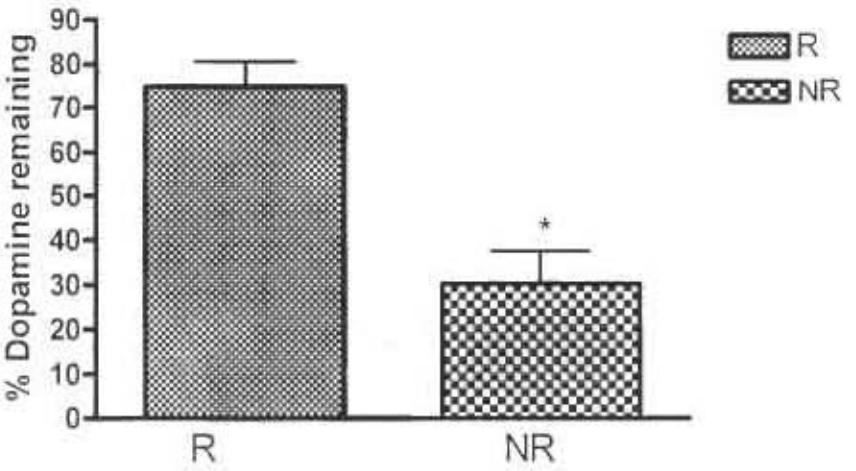


Figure 2.4.5.2 Percentage of striatal dopamine remaining in the lesioned hemisphere of the R, n=9 and NR, n=9 rats. Data reported in Table 2.4.5.2.

2.4.5.3 DOPAC concentration

There was no significant difference between the DOPAC concentration in the lesioned or non-lesioned hemispheres in all the rats *(Table 2.4.5.3., Figure 2.4.5.3)*.

There was a significant difference between the percentage of striatal DOPAC remaining in the lesioned hemisphere of rats (R) that had access to running wheels and the percentage of striatal DOPAC in the lesioned hemisphere of rats (NR) in cages with immobilised running wheels *(Table 2.4.5.3, Figure 2.4.5.3.1)*. The percentage of striatal DOPAC in the lesioned hemisphere was calculated as the amount of DOPAC present in the lesioned hemisphere of rats relative to the amount of DOPAC present in the non-lesioned hemisphere.

2.4.5.2 Amount of striatal dopamine remaining in the lesioned hemisphere

There was a significant difference between the percentage of striatal dopamine remaining in the lesioned hemisphere of rats (R) that had running wheels attached and the percentage of striatal dopamine in the lesioned hemisphere of rats (NR) in cages with immobilised running wheels *(Table 2.4.5.2, Figure 2.4.5.2)*. The percentage of striatal dopamine in the lesioned hemisphere was calculated as the amount of dopamine present in the lesioned hemisphere of rats relative to the amount of dopamine present in the non-lesioned hemisphere.

Table 2.4.5.2 The percentage of striatal dopamine remaining in the lesioned hemisphere of rats with access to running wheels and the percentage of striatal dopamine remaining in the lesioned hemisphere of rats in cages with immobilised wheels (NR). *(R vs NR $p<0.001$)

Dopamine concentration (ng/g wet weight).			
DA non-lesion (R)	DA lesion (R)	DA non-lesion (NR)	DA lesion (NR)
1961 ± 650	1463 ± 390	3870 ± 497	1168 ± 363

% Dopamine remaining in the lesioned hemisphere.	
R	NR
74.6 ± 6	30.2 ± 7.3*

Table 2.4.5.1 Dopamine concentration in the striatum of rats that had access to running wheels (R) attached and rats that had cages with immobilised running wheels (NR). Dopamine concentration was measured in the non-lesioned and lesioned hemispheres of the rats. *(DA non-lesion (NR) vs DA non-lesion (R), $p<0.01$) and **(DA non-lesion (NR) vs DA lesion (NR), $p<0.01$).

Dopamine concentration (ng/g wet weight)			
DA non-lesion (R)	DA lesion (R)	DA non-lesion (NR)	DA lesion (NR)
1961 ± 650 *	1463 ± 390	3870 ± 497	1168 ± 363**

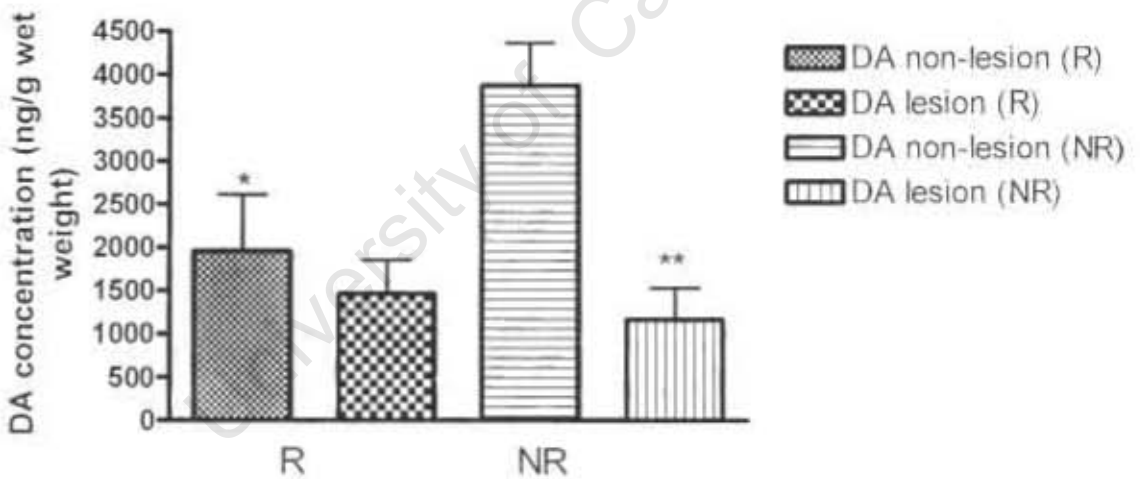


Figure 2.4.5.1 Dopamine concentration in the striatum of rats that had access to running wheels (R, n=9) and rats that had cages with immobilised running wheels (NR, n=9). Dopamine concentration was measured in the non-lesioned and lesioned hemispheres of the rats. . *(DA non-lesion (NR) vs DA non-lesion (R), $p<0.01$) and **(DA non-lesion (NR) vs DA lesion (NR), $p<0.01$). Data reported in Table 2.4.5.1.

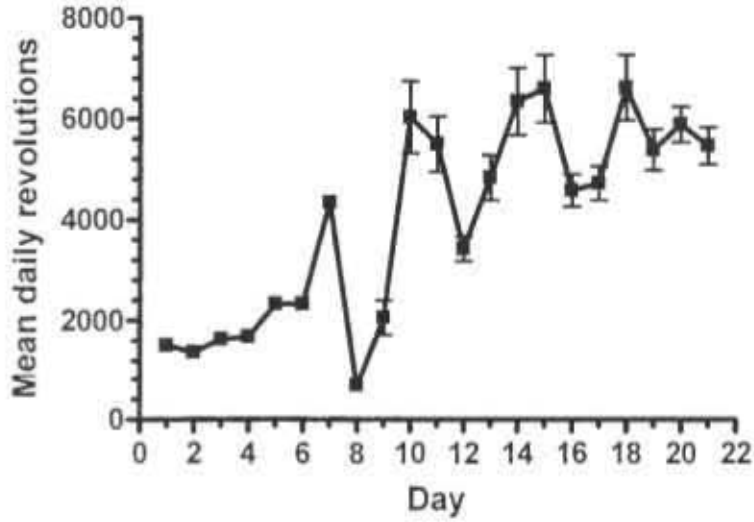


Figure 2.4.4.1 Mean number of revolutions performed by the rats in the cages with running wheels (n=9) plotted against days spent in the cages. Data reported in Table 2.4.4.1

2.4.5 Quantification of striatal dopamine and its metabolite DOPAC.

2.4.5.1 Dopamine concentration

The striatal dopamine concentration in the non-lesioned rats in cages with immobilised running wheels (NR), was significantly greater than the dopamine concentration in the lesioned hemisphere of the NR rats *(Table 2.4.5.1, Figure 2.4.5.1)*. The dopamine concentration in the non-lesioned hemisphere of the NR rats was significantly greater than the dopamine concentration in the non-lesioned hemisphere of the rats that had access to running wheels (R) *(Table 2.4.5.1, Figure 2.5.4.1)*. However there was no significant difference between the dopamine concentration of the intact hemisphere and the lesioned hemisphere of the R rats *(Table 2.4.5.1, Figure 2.4.5.1)*. There was no significant difference between the lesioned hemisphere of the rats with access to running wheels and the lesioned hemisphere of the rats that had their running wheels immobilized.

2.4.4 HPLC analysis

A second group of rats were used to determine striatal dopamine and DOPAC concentrations.

2.4.4.1 Locomotor activity

Running wheel revolutions increased from day 1 until the day of the lesion 7 days later *(Table 2.4.4.1, Figure 2.4.4.1)*. Following stereotaxic infusion of 6-OHDA, there was a dramatic decline in wheel revolutions on day 8 *(Table 2.4.4.1, Figure 2.4.4.1)*. It took the rats 3 days before they began to run at pre lesion revolutions (day10) *(Table 2.4.4.1, Figure 2.4.4.1)*.

Table 2.4.4.1 Mean daily distance run by the rats pre lesion (day 1 to 7) and post lesion (day 8 to 21).

Day	Daily distance run (m)
1	1510 ± 68.3
2	4342 ± 146
8	680 ± 140
10	6044 ± 719
21	5488 ± 367

Table 2.4.3 Tyrosine hydroxylase positive cells in the substantia nigra of lesioned hemispheres expressed as a percentage of the number of tyrosine hydroxylase positive cells in the non-lesioned hemispheres of rats that had access to running wheels (R) and rats that were in cages with immobilised running wheels(NR). *(R vs NR, $p<0.005$)

% Tyrosine hydroxylase positive cells in the lesioned hemisphere.	
R	*NR
64.89 ± 2.80	86.15 ± 6.72

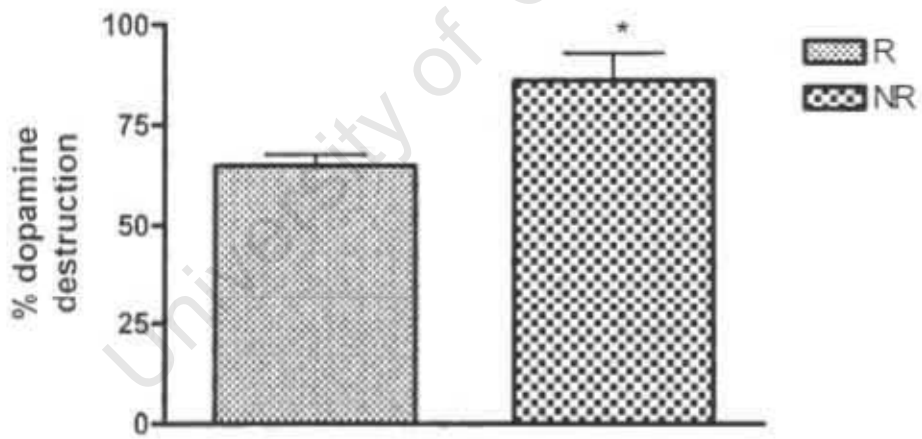


Figure 2.4.3 Tyrosine hydroxylase positive cells in the substantia nigra of lesioned hemispheres expressed as a percentage of tyrosine hydroxylase positive cells in the non-lesioned hemispheres of rats that had access to running wheels (R, n=14) and rats that had their wheels immobilised (NR, n=13). *(R vs NR, $p<0.005$). Data reported in Table 2.4.3.

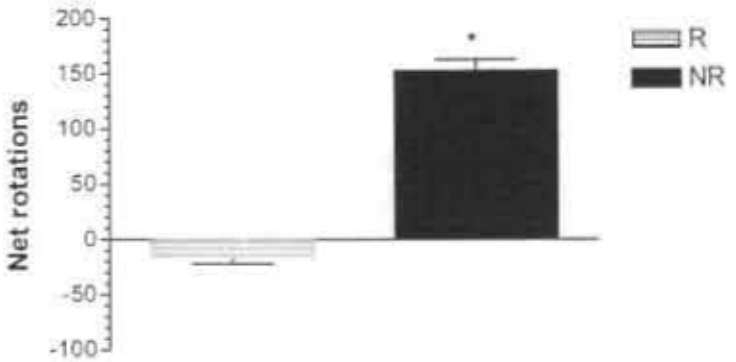


Figure 2.4.2 Apomorphine-induced rotations of rats that had access to free running wheels (R, n=14) and rats that were in cages with immobilised running wheels (NR, n=13) following subcutaneous apomorphine injections. * (R vs NR, $p < 0.001$). Data reported in Table 2.4.2.

2.4.3 Tyrosine Hydroxylase immunohistochemistry.

There was a significant difference between the percentage of dopamine neuron destruction in the substantia nigra of rats with access to running wheels (R) and percentage of dopamine neuron destruction in the substantia nigra of rats (NR) in cages with immobilised running wheels *(Table 2.4.3, Figure 2.4.3)*. Dopamine neuron destruction was calculated as the number of tyrosine hydroxylase positive cells present in the lesioned hemisphere expressed as a percentage of tyrosine hydroxylase positive cells in the non-lesioned hemisphere.

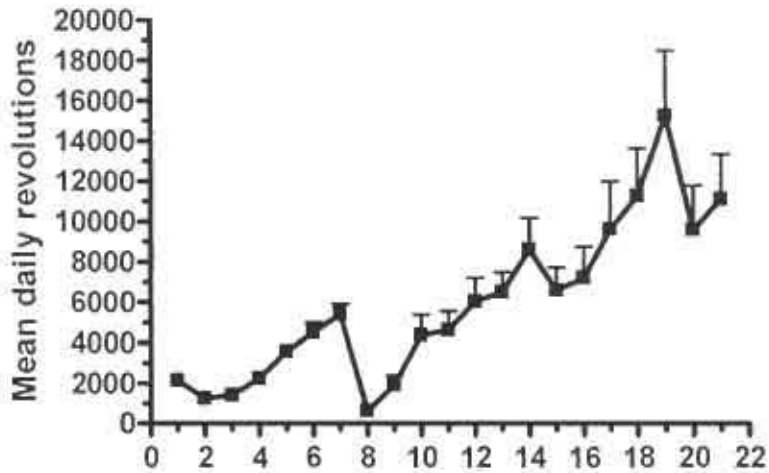


Figure 2.4.1 mean daily distance run by the rats (n=14) plotted against days spent in the cages. Data reported in Table 2.4.1.

2.4.2 Apomorphine-induced rotations

The rats that had access to free running wheels (R) did not rotate contralaterally in response to apomorphine injection *(Table 2.4.2, Figure 2.4.2)*. The rats in cages with immobilised running wheels (NR) performed significantly more contralateral rotations in response to apomorphine injection than the R rats *(Table 2.4.2, Figure 2.4.2)*.

Table 2.4.2 Apomorphine-induced rotations of rats that had access to free running wheels (R) and rats that were in cages with immobilised running wheels (NR) following subcutaneous apomorphine injections. * (R vs NR, $p < 0.001$)

Net rotations after 90 min in a rotometer.	
R	NR
-15.89 ± 2.80	* 153 ± 4.76

2.4 RESULTS

2.4.1 Locomotor activity

Running wheel revolutions recorded from rats in the free running wheels increased steadily from day 1 to day 7 *(Table 2.4.1, Figure 2.4.1)*. On day 7 the rats were lesioned and then returned to their cages, 2 hours after the stereotaxic surgery. The activity in the running wheels decreased on day 8 following the surgery but increased steadily thereafter and returned to pre-surgery revolutions on day 11.

Table 2.4.1 Mean daily distance run by the rats pre lesion (day 1 to 7) and post lesion (day 8 to 21).

Day	Distance travelled (m)
1	2135 ± 206
7	5388 ± 524
8	619 ± 229
11	4608 ± 931
21	11077 ± 2214

2.6 DISCUSSION

In this study experimental rats were allowed free access to running wheels. No stimuli were applied to force the rats to exercise. The results of this study suggest that rats that had free access to a running wheel for 7 days prior to unilateral 6-OHDA injection into the MFB and again for 14 days post lesion (beginning 2 hours post lesion) showed neuroprotection of the dopamine neurons after voluntary exercise. This is based on the reduced number of apomorphine-induced contralateral rotations and the reduced dopamine neuron destruction in the lesioned substantia nigra in rats that had access to running wheels.

There was a steady increase in the mean number of daily revolutions between days 1 and 7. It is important to note that in general the running behaviour of the rats pre and post stereotaxic surgery remained similar i.e. there was a progressive increase in the number of wheel revolutions. The decrease in the number of revolutions of the running wheels on days 8 and 9 can be due to two factors. On the day of the lesion the rats were taken out of their cages just prior to the commencement of their dark cycle. They were only returned to their cages two hours after completion of the surgery. This period took between 3 to 6 hours depending on when the rat was lesioned. Secondly, the surgery itself was a traumatic event and as a result there was a decline in the physical activity of the animals during the recovery phase. What is important is that the experimental group (free access to running wheels) took at most three days before normal daily running activity was resumed. It is also important to note that the rats in the cages with immobilised wheels were not themselves immobilised. Therefore the changes that occurred were not a result of immobilisation or disuse. They were due to an increase in exercise in the experimental group rather than a complete lack of exercise in the control group.

For the dopamine receptor agonist apomorphine to induce contralateral rotations, it has been suggested that the dopamine deficit in the striatum

ipsilateral to the lesion should be greater than 70% after a unilateral 6-OHDA lesion of dopamine neurons *(Hefti et al 1980, Dunnett et al 1988, Hudson et al 1993)*. In the present investigation we showed that rats that had access to free running wheels did not rotate contralaterally post injection with apomorphine. In contrast, rats that had their running wheels immobilised demonstrated more severe lesions with apomorphine injections inducing vigorous contralateral rotations in these rats. In rats, contralateral rotations are thought to be due to stimulation of the supersensitised dopamine receptors in the lesioned hemisphere resulting in turning towards the non-lesioned side *(Hudson et al 1993)*. This suggests that in the present study, dopamine receptors in the lesioned hemisphere were not upregulated or supersensitised because fewer dopamine neurons degenerated during the lesioning process. Contralateral rotations induced by apomorphine (dopamine receptor agonist) are only observed in animals that have severe ipsilateral depletion of dopamine innervation of the striatum and is generally used as a marker of over 70% striatal dopamine depletion after a unilateral 6-OHDA lesion of dopamine neurons *(Hudson et al, 1993)*. In the present study, we used an apomorphine concentration of 0.5 mg/kg and we did not induce significant contralateral rotations in the rats with access to running wheels. Therefore there appears to be sufficient dopamine neurons to allow normal activity patterns in the experimental rats.

There was 65% destruction of tyrosine hydroxylase positive cells in the lesioned substantia nigra of rats that had access to running wheels and 86 % destruction in the lesioned hemisphere of rats that had cages with immobilised running wheels compared to the non-lesioned hemisphere. The absence of apomorphine-induced rotations and the lesser dopamine neuron destruction in the lesioned substantia nigra in rats that were exercised suggests that exercise provided neuroprotection to the dopamine neurons. HPLC quantification of striatal dopamine concentration in the rats that had access to running wheels showed the striatal dopamine concentration in the lesioned hemisphere was 74.6% of dopamine in the non-lesioned striatum (25.4% difference) whereas there was 30% striatal dopamine remaining following 6-OHDA infusion in rats in cages with immobilised running wheels. For destruction in the nigrostriatal pathway to be considered a model of

Parkinson's disease, there should be 70 to 80% dopamine depletion in the striatum *(Bjorklund et al 2000, Dauer et al 2003)*. However the dopamine concentration in both hemispheres of the rats with access to running wheels was significantly lower than the dopamine concentration in the non-lesioned hemisphere of the rats with immobilised running wheels and not significantly different from the dopamine concentration in the lesioned hemisphere of the rats in the immobilised wheels. This might suggest that the dopamine concentration in the non-lesioned hemisphere of the exercised rats is decreased to the same level as the concentration in the lesioned hemisphere so as to maintain bilateral symmetry and coordination during voluntary exercise. Dopaamine / DOPAC ratio's are used as a measure of the rate of dopamine turnover with ratio's greater than 1 suggesting that there is an increase in dopamine synthesis and release *(Tillerson et al 2001)*. In the *Tillerson et al (2001)* model, forced exercise immediately following 6-OHDA infusion resulted in complete sparing of dopamine neurons in the lesioned hemisphere with a striatal dopamine / DOPAC ratio of 1. In the present study, the dopamine / DOPAC ratio in the lesioned hemispheres of the rats with access to running wheels and in cages with immobilised running wheels was raised but not significantly different from each other. Although both greater than I, there was also no significant difference between the dopamine turnover rate in the lesioned and non-lesioned hemispheres of the rats with access to running. However the dopamine turnover rate in the non-lesioned hemisphere of the non exercising rats was significantly greater than in the lesioned hemisphere. This suggests that in excessive dopamine neuron destruction, the decrease in dopamine supply to the terminals in the striatum does not only result in sensitisation of the post synaptic dopamine receptors in the lesioned striatum but might also increase dopamine synthesis in the non-lesioned hemisphere. Therefore the injection of the dopamine agonist apomorphine does not have a significant impact on the non-lesioned hemisphere which is dopamine replete. In the rats with access to running wheels, there was more than 60% dopamine neurone destruction in the lesioned substantia nigra and 25% depletion in striatal dopamine suggesting that complete sparing of dopamine neurons did not occur hence the increased dopamine turnover rate. However the dopamine turnover rate is similar in the

lesioned and non-lesioned hemisphere suggesting symmetry in dopamine release hence the absence of apomorphine induced rotations. The results of this study suggest that 6-OHDA injected into the MFB results in an increase in the dopamine turnover in both the lesioned and the non-lesioned hemispheres. Initially, with mild loss of dopamine terminals, the remaining neurons are able to compensate for the loss of dopamine innervation of the striatum. However, when degeneration of dopamine neurons increases to more than 50%, the remaining dopamine neurons in the substantia nigra are no longer able to compensate for the loss and Parkinsonian symptoms develop despite post-synaptic receptor supersensitivity *(Ungerstedt 1971, Hudson et al 1993)*.

The present findings demonstrates that voluntary physical exercise performed at the rats' own pace can have neuroprotective effects on the motor functions of the rats after lesioning with a neurotoxin 6-OHDA. The absence of contralateral rotations in the presence of high doses of apomorphine suggests that there is enough dopamine to prevent upregulation and supersensitisation of dopamine receptors in the striatum. The present findings may have implications for the treatment of patients with Parkinson's disease as exercise induced neuroprotection can reduce degeneration of the dopamine neurons in the substantia nigra and terminals in the striatum. Therefore exercise may be beneficial to patients with Parkinson's disease and reduce the level of symptoms in these patients

2.7 CONCLUSION

Studies that have demonstrated neuroprotection in a Parkinsonian rat model relied mostly on forcing the rat to exercise the injured forelimb post unilateral 6-OHDA injection *(Tillerson et al 2001)*. Other studies that also examined neuroprotection following a brain insult forced the rats to exercise on a treadmill *(Carro et al 2001)*. In our study the fact that rats in the immobilised wheels did have access to the wheel but could not run raises a question of whether there is an optimum amount of exercise that a rat should have in

order to protect the dopamine neurons. In addition to neuroprotection there is also evidence of adaptation such as the decrease in non-lesioned hemisphere striatal dopamine which accounts for the absence of asymmetrical behaviour associated with apomorphine injection in unilaterally 6-OHDA infused rats that had access to running wheels. Exposure to free running wheels results in greater dopamine neuron sparing as shown by dopamine neuron destruction in the substantia nigra of the lesioned hemisphere. Therefore exposure to free running wheels appears to be important as a neuroprotective effect in rats that have been lesioned to mimic Parkinson's disease. This could be an important factor in prescribing/planning an exercise regimen for Parkinson's disease patients.

CHAPTER 3

The effects of stress on brain GDNF of spontaneously running rats.

3.1 INTRODUCTION

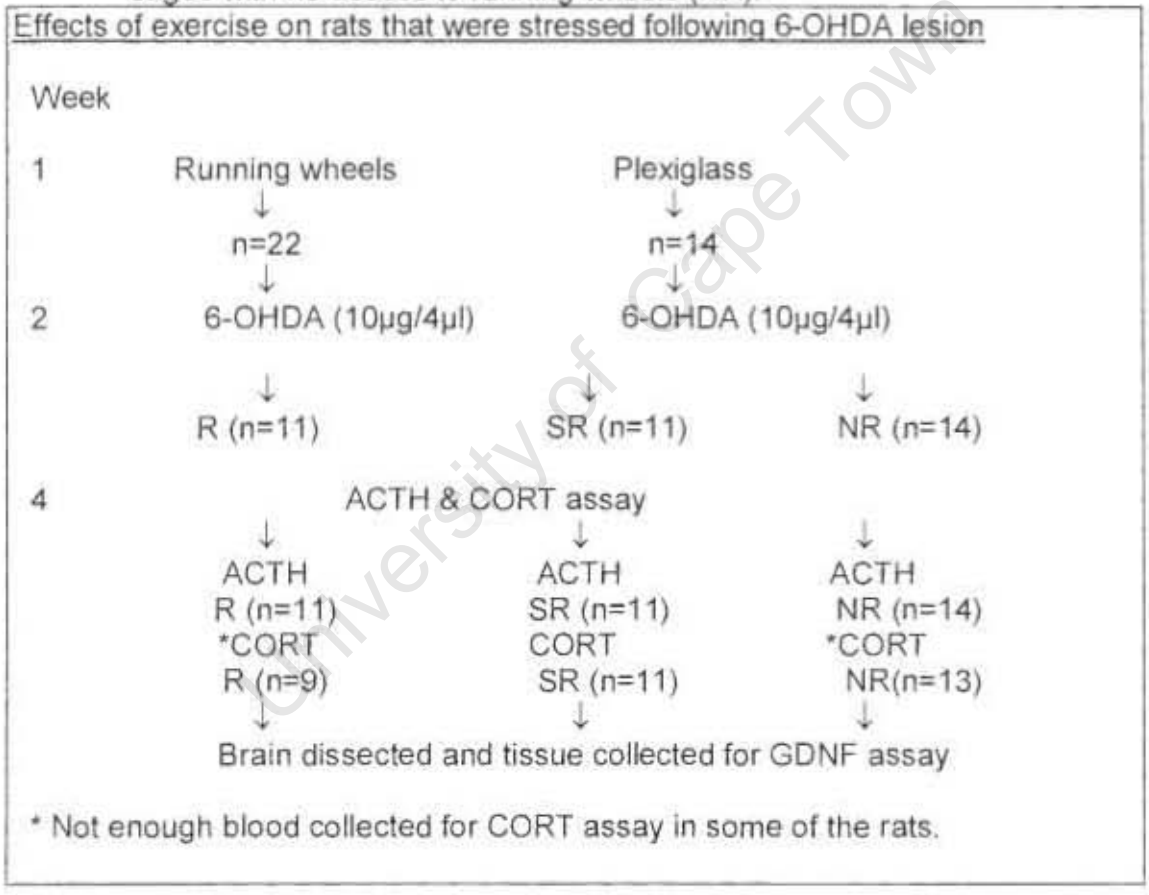
Following MFB lesions with 6-OHDA, nigrostriatal signalling may be maintained by reduced dopamine reuptake, increased dopamine synthesis and release and proliferation of dopamine receptors *(SECTION 1.2)*. These changes are thought to mimic preclinical Parkinson's disease symptoms in a Parkinsonian rat model *(Tillerson et al 2001)*. When the dopamine degeneration becomes too great, the compensatory effects become inadequate and pharmacological treatment using direct or indirect dopamine agonists becomes necessary *(Tillerson et al 2002, O'Dell et al 2007)*. However, pharmacological therapies are often short term as drug-induced side effects limit continued use *(O"Dell et al 2007)*. One prospective non-pharmacological therapy for Parkinson' disease patients, is exercise which has been shown to increase motor and cognitive functions in diseased patients *(Nieuwboer et al 2001)*. Studies have shown that exercise can increase the expression of GDNF mRNA and other neurotrophic factors in areas of the rat brain such as the hippocampus *(Neeper et al 1996)*. In the nigrostriatal area studies focusing on the neuroprotective effects of GDNF have mainly focused on infusing GDNF into the area that is subsequently lesioned with 6-OHDA *(SECTION 1.5.2)*. Studies that have focused on the neuroprotective effects of exercise in 6-OHDA lesioned rats have mainly forced the rats to exercise either by treadmill running or by casting (forelimb cast) the uninjured limb immediately after 6-OHDA infusion thus forcing the rats to use the injured limb *(SECTION 1.4)*.The reliance on forced exercise in these models may introduce the confounding factor of stress *(O'Dell et al 2007)*. Stress in the form of increased glucocorticoids such as corticosterone has been shown to decrease the expression of neurotrophic factors such as BDNF *(SECTION 1.5.3)*.

This raises the question of whether introducing a stress protocol to a Parkinsonian rat model will inhibit the beneficial effects of exercise. Therefore our aim was to investigate whether introducing stressors to 6-OHDA lesioned rats will increase the concentration of corticosterone in plasma and whether this increase will result in decreased concentrations of GDNF in the striatum, substantia nigra and the VTA.

3.2 MATERIALS AND METHODS

Thirty six Long Evans rats that weighed between 220-250 grams were placed in a room with a 12 h (11 am to 11pm) dark/light cycle in the Departmental Animal house facility. There were 4 rats per cage.

Table 3.2 Flow diagram of experimental protocol with rats that had access to running wheels (R), rats that had access to running wheels and received various stressors following lesion (SR) and rats that were in plexiglass cages with no access to running wheels (NR).



3.2.1 Running Experiments

One week later the rats were weighed and divided into two groups viz. the runners and non-runners. The runners were placed individually into cages with running wheels and the non-runners were placed individually in cages without running wheels *(Table 3.2)*. Running revolutions were recorded daily between 10 am and 11 am (which was the hour before the rats entered the dark cycle). One week after the rats were placed into their respective cages, they were weighed and taken to a surgical laboratory in preparation for 6-OHDA lesion. The rats were moved to the surgical lab at least one hour before surgery started so as to acclimatize to the new environment.

3.2.2 Stereotaxic surgery.

The infusion of 6-OHDA was performed as described in Section 2.2.2.

3.2.3 Stress protocol for the stressed runners (SR rats)

The day after lesioning with 6-OHDA, the SR rats were subjected to daily 1 h wheel immobilization from 10h45 to 11h45. Rats are more active in the dark cycle so immobilizing the wheels before the commencement of the dark cycle was to ensure that the rats could not run thus stressing them. A single additional stress three days after lesioning was introduced when the rats were subjected to a 7 h shift in the light dark cycle by placing them in a room with a 6 am to 6 pm light/dark cycle. Eleven days after lesioning, the rats were subjected to a single 24 h food deprivation. The rats in the R and NR groups received food and water *ad libidum* and were kept under standard Animal house conditions (temp, 21-24°C). The running revolutions made by the rats with access to free running wheels were recorded daily for a further two weeks after stereotaxic surgery.

3.2.4 Trunk blood and brain tissue collection.

Twenty one days after the rats were initially placed into their individual cages, the rats were weighed and taken to the room where behavioural tests were performed. The rats were taken to the behavioural room at least one hour before trunk blood was collected. Following decapitation, trunk blood was collected in vials lined with EDTA to prevent clotting. The vials were spun at 15000 RPM in a 4 °C centrifuge for 15 min after which the plasma was collected and stored at -80°C until the radioimmunoassay were performed. To remove the brain, the skull was removed with the aid of bone cutters. The brain was scooped from the calverium and placed in ice cold 0.9% saline so as to slow down protease enzyme activity. The brain was cut in half and the striatum was scooped out and weighed. The striatum was immediately frozen at -80°C following weighing. This was followed by dissection of the substantia nigra and VTA. Following weighing the substantia nigra and VTA were frozen at -80°C in preparation for GDNF analysis.

3.2.5 Radioimmunoassays

3.2.5.1 ACTH Assay

A two-site solid phase immunoradiometric assay (IRMA) kit, EURIA-ACTH c.t. (EURO-DIAGNOSTICA,Malmö, Sweden), was used to determine the amount of adrenocorticotropic hormone (ACTH) in the plasma of the rats.

The tubes to which the samples, standards, total counts (TC) and controls were to be added were labelled in duplicate. The 2 controls and 7 standards (A-G) were reconstituted by adding 1 ml of distilled water. The radioactive ACTH antibody tracer was reconstituted by adding 12.4 ml distilled water. Thirty min passed before the reconstituted materials were ready for use. Two hundred microliters of each sample, standard and control were pipetted into the appropriately labelled tube. All tubes including the TC tubes received 200 µl of

the radioactive antibody tracer. The tubes were mixed thoroughly using a vortex mixer and then incubated overnight at room temperature. Each tube except the TC tubes was washed twice with 2 ml of wash buffer. After aspirating the wash buffer thoroughly the radioactivity in the tubes and TC tubes was counted using a gamma counter. A standard curve was generated by the standards and the concentration of ACTH in each sample was calculated by interpolation of unknown values against the standard curve generated.

3.2.5.2 Corticosterone assay

Plasma corticosterone levels were measured using an ImmuChem double antibody ^{125}I Corticosterone RIA kit (MP Biomedicals, LLC, Orangeburg,NY).
The sample tubes were labelled in triplicate. Another set of tubes prepared in duplicate was labelled for NSB, 2 control and 7 corticosterone standards (0, 25, 50, 100, 250, 500,1000 ng/ml). Two milliliters of steroid diluent was added to one of the set of tubes prepared for the samples. Ten microliters (µl) of sample was added to the tubes with the steroid diluent and 300 µl of steroid diluent was added to the tubes marked NSB. One Hundred microliters of steroid diluent was added to the tubes marked 0ng/ml. To reconstitute the control samples, 2ml of distilled water was added. A 100 µl aliquot of corticosterone standard was added to the appropriately marked tube (25 ng/ml -1000 ng/ml) and 100 µl of the reconstituted controls was added to the control tubes. The sample and diluent mixture prepared earlier (100 µl) was added in duplicate to the remaining sample tubes followed by 200 µl of radioactive corticosterone tracer. This was followed by 200 µl of anti-serum into all the tubes except the NSB tubes. After thoroughly mixing using a vortex mixer, 500 µl of precipitant solution was added to the tubes. The tubes were thoroughly mixed and centrifuged at 2500 rpm for 15 min. The supernatant was aspirated and the corticosterone radioactivity was measured using a gamma counter.
The procedure followed was as outlined in the kit manual; a standard curve was generated using the standards provided and corticosterone concentration was

calculated by interpolation of unknown values against the standard curve generated.

3.2.6 GDNF ELISA

3.2.6.1 Sample preparation

The tissue samples were taken out of the -80°C freezer and diluted to 20 times their wet weight with lysis buffer (20µl for each mg of tissue). The lysis buffer recipe included the lysis core buffer ingredients, phosphorylase inhibitors (sodium orthovanidate, sodium fluoride and sodium pyrophosphate) and the protease inhibitor complex; Complete (Roche Diagnostics, GmbH, Mannheim, Germany). The lysis buffer sample mixture was homogenised by sonication and centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was collected and underwent an acid treatment procedure by adding Dulbecco's phosphate buffered saline (DPBS) (1 in 4 dilution) and 4 µl of 1 M HCL. The samples were mixed for 15 min in a haematological mixer. Following the acid treatment procedure 4 µl of 1 M NaOH was added to each sample to make the pH basic. The treated samples were stored at -80°C while the 96 well microplates were being prepared.

3.2.6.2 ELISA assay

The 96 well microplates were incubated in Coating Buffer A (Biosource, Camarillo, California) for 12 to 18 h. The plates were then washed with wash buffer (Biosource, Camarillo, California) and incubated in blocking buffer (NaCl, Na2HPO4, KH2PO4, KCl, bovine serum albumin fraction V) for 1 h. Eight standards (serial 1:2 dilutions from 500 pg/ml to 7.8 pg/ml) (Biosource, Camarillo, California) and samples were pipetted in duplicate into the wells. The 8th standard was assay buffer (Biosource, Camarillo, California), therefore in each 96 well microplate there were 8 standards and 40 samples. Detection buffer

(Biosource, Camarillo, California) was added immediately afterwards into each well. The plates were incubated for 2 h while continually shaking in a plate shaker (700 rpm). Following incubation, the plates were washed 4 times with wash buffer and then incubated with streptavidin-HRP (Biosource, Camarillo, California) for 30 min in a plate shaker (700 rpm). The plates were washed (4 times) after which they were incubated for 30 min with the chromogen 3, 3', 5, 5' Tetramethylbenzidine (TMB) while continually shaking at 700 rpm in a plate shaker. After the 30 min of incubation, 1.8 M sulphuric acid was added to the plates to stop the colour reaction and absorbance was measured at 450 nm (reference absorbance:650 nm) in an illuminomitor before 30 min elapsed.

3.3 STATISTICAL ANALYSIS

Graph Pad Prism 4 was used for statistical analysis. ANOVA was used to analyse the data and when significant differences were found ($p<0.05$), post hoc comparison using Tukey's Multiple Comparison Test was performed. Results are reported as mean ± standard error of the mean (SEM).

3.4 RESULTS

3.4.1 Locomotor activity

The daily running revolutions increased from day 1 until the day of the lesion 7 days later *(Table 3.4.1, Figure 3.4.1)*. Following stereotaxic lesioning with 6-OHDA, there was a dramatic decline in daily running revolutions in both the R and SR groups. It took the R rats 4 days to run at pre lesion revolutions (day 11) and the SR rats 3 days (day 10) *(Table 3.4.1, Figure 3.4.1)*. On day 18 there was a dramatic increase in the running revolutions of the SR rats which decreased to pre food deprivation levels afterwards *(Table 3.4.1, Figure 3.4.1)*.

Table 3.4.1 Mean daily distance run by the rats in running wheels (R) and rats in running wheels that were exposed to various stressors following lesion (SR).

	Daily Distance traveled (m)	
Day	R	SR
1	1656 ± 280	756 ± 489
7	2497 ± 482	4549 ± 701
8	459 ± 245	604 ± 122
10	1611 ± 408	4110 ± 770
11	1896 ± 564	3350 ± 226
18	6234 ± 1819	15542 ± 1176
21	4190 ± 1355	5425 ± 1939

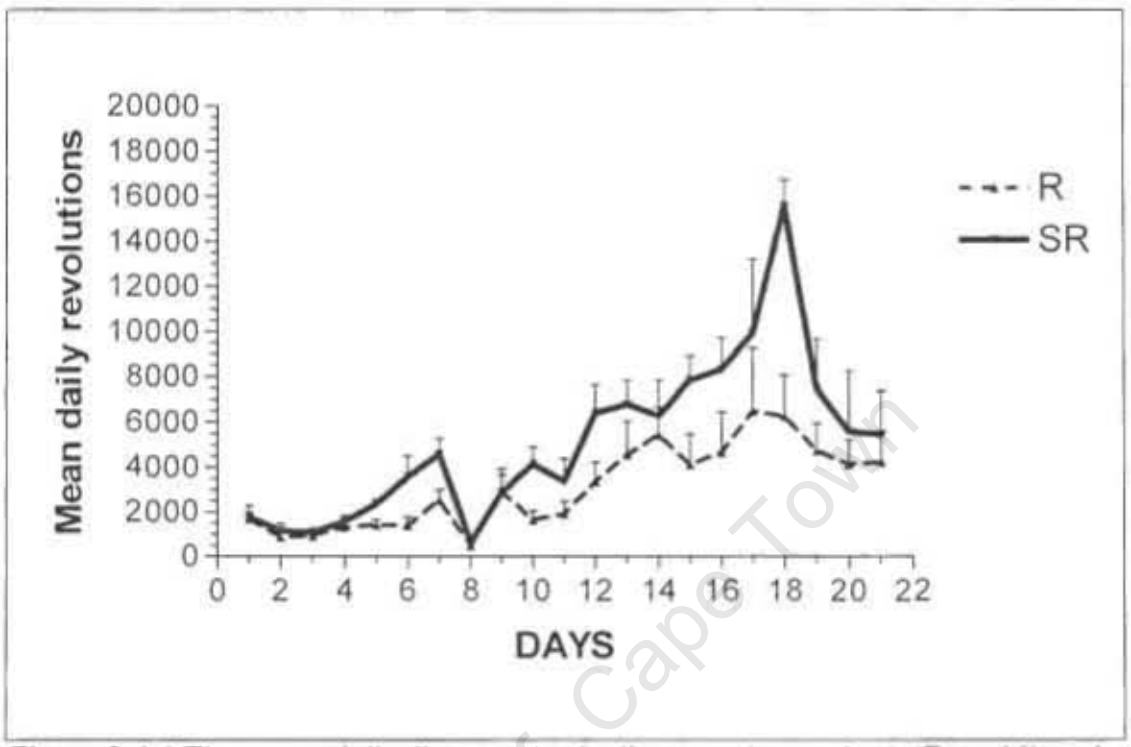


Figure 3.4.1 The mean daily distance run by the non-stressed rats(R, n=11) and rats that were stressed following lesion (SR, n=11). Data reported in Table 3.4.1

3.4.2 Rat weights

There was no significant difference between the weekly rat weights in all groups (NR, S, SR) *(Table 3.4.2, Figure 3.4.2)*. There was a steady increase in the weights of the rats from week 1 to week 4 *(Table3. 4.2, Figure3.4.2)*.

Table 3.4.2 Weight of the rats before lesion (week 1), day of lesion (week 2), one week after lesion and on the day of trunk blood collection.

	Weight in (g)		
Week	NR	R	SR
1	270 ± 10.5	267 ± 9.37	252 ± 6.58
2	289 ± 17.1	302 ± 6.0	289 ± 12.8
3	321 ± 23.0	323 ± 4.84	311 ± 12.4
4	332 ± 34.3	351± 6.00	330 ± 9.88

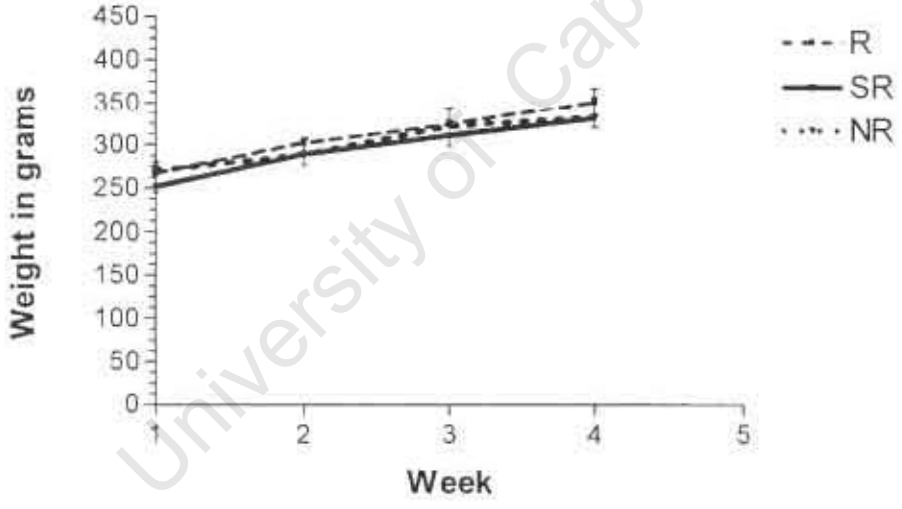


Figure 3.4.2 The mean weights of the rats in the running wheels (R, n=11), the rats in the plexiglass cages (NR, n=14) and the rats in the running wheels that were stressed (SR, n=11). Data reported in Table 3.4.2

3.4.3 ACTH analysis

The basal plasma ACTH concentration of the R rats was significantly higher than the basal ACTH concentration of the NR rats *(Table 3.4.3, Figure 3.4.3)*. There

was no significant difference between the basal ACTH concentration of the SR rats and the basal ACTH concentration of the NR rats and R rats *(Table 3.4.3, Figure 3.4.3)*.

Table 3.4.3 Plasma ACTH concentration in lesioned rats(NR) that were in plexiglass cages, rats (R) that had access to running wheels and stressed rats (SR) that had access to running wheels. *(NR vs R, $p<0.05$).

Basal Plasma ACTH concentration (pg/ml)		
NR	R	SR
83.6 ± 9.78	125 ± 18.6*	91.7 ± 11.3

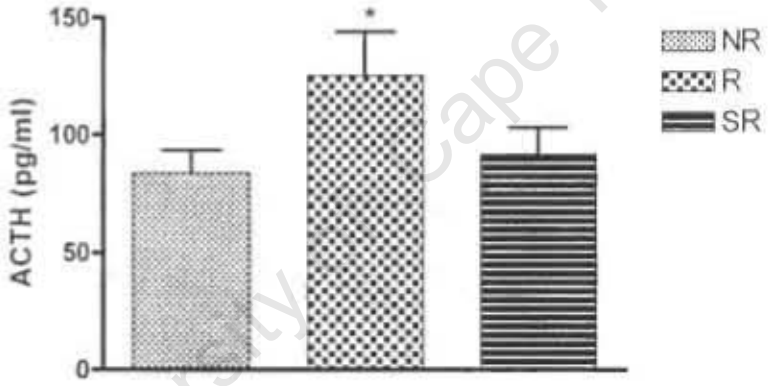


Figure 3.4.3 Basal concentration of ACTH in rats in plexiglass cages (NR, n=14), rats in running wheels (R, n=11) and rats that were stressed (SR, n=11). *(NR vs R, $p<0.05$). Data reported in Table 3.4.3.

3.4.4 Corticosterone (CORT) analysis

The basal corticosterone concentrations of the R and SR rats were significantly higher than the basal corticosterone concentration of the NR rats *(Table 3.4.4, Figure 3.4.4)*. There was no significant difference between the basal corticosterone concentration of the R and SR rats.

Table 3.4.4 Plasma corticosterone concentration in lesioned (NR) rats that were in plexiglass cages, rats (R) that had access to running wheels and stressed rats (SR) that had access to running wheels. *(NR vs R, $p<0.01$) and **(NR vs SR, $p<0.05$).

Plasma corticosterone levels (pg/ml)		
NR	R	SR
117 ± 28.7	347 ± 33.3*	294 ± 74.3**

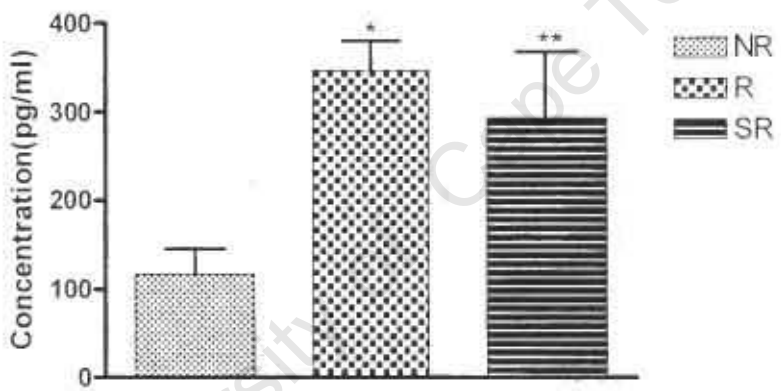


Figure 3.4.4 Plasma corticosterone concentration in rats in plexiglass cages (NR, n=13), rats in cages with running wheels (R, n=9) and rats in cages with running wheels that were stressed (SR, n=11). *(NR vs R, $p<0.01$) and **(NR vs SR, $p<0.05$). Data reported in Table 3.4.4

3.4.5 GDNF ELISA

3.4.5.1 GDNF concentration in the striatum

There was no significant difference between the GDNF concentration in the striatum of the ipsilateral (Left) and contralateral (Right) hemispheres of the rats following unilateral 6-OHDA infusion *(Table 3.4.5.1, Figure 3.4.5.1).*

Table 3.4.5.1 GDNF concentration in lesioned (NR) rats that were in plexiglass cages, rats (R) that had access to running wheels and stressed rats (SR) that had access to running wheels.*(NR (SN) Right VS SR (SN) Right, $P<0.05$.

GDNF concentration (pg/mg wet weight)					
NR					
Striatum		SN		VTA	
Left	Right	Left	Right	Left	Right
57 ± 8.93	48 ± 7.19	312 ± 23.41	325 ± 29.98	332 ± 80	301 ± 91.88
R					
Striatum		SN		VTA	
Left	Right	Left	Right	Left	Right
65 ± 10.94	57 ± 32.62	246 ± 46.76	178 ± 36.68	368 ± 38.04	279 ± 28.01
SR					
Striatum		SN		VTA	
Left	Right	Left	Right	Left	Right
38 ± 11.68	30 ± 9.49	232 ± 53.93	143 ± 40.07*	241 ± 89.98	205 ± 73.55

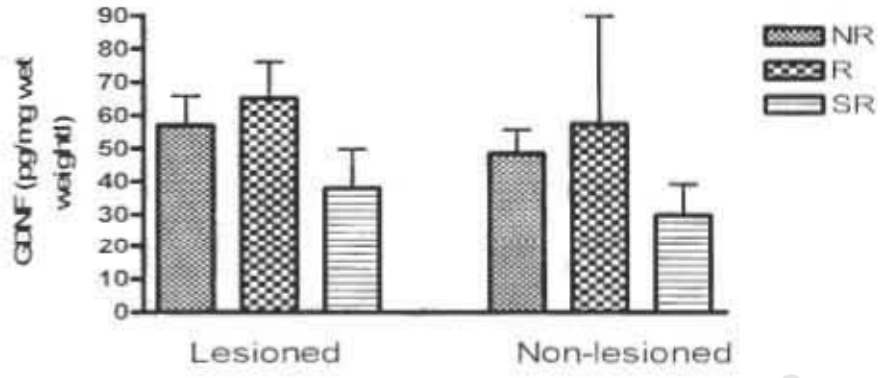


Figure 3.4.5.1 GDNF concentration in the striatum of lesioned and non-lesioned striatum of rats in plexiglass cages (NR, n=14), rats with running wheels (R, n=11) and rats with running wheels that were stressed (SR, n=11). Data reported in Table 3.4.5.1

3.4.5.2 GDNF concentration in the substantia nigra

The GDNF concentration in the substantia nigra of the non-lesioned hemisphere of NR rats was significantly more than the GDNF concentration in the non-lesioned hemisphere of SR rats *(Table 3.4.5.1, Figure 3.4.5.2)*. There was no significant difference between the GDNF concentration in the lesioned hemispheres of the NR, R and SR rats *(Table 3.4.5.1, Figure 3.4.5.2)*. There was no significant difference between the GDNF concentration in the non-lesioned hemispheres of the NR and R rats or R and SR rats *(Table 3.4.5.1, Figure 3.4.5.2)*.

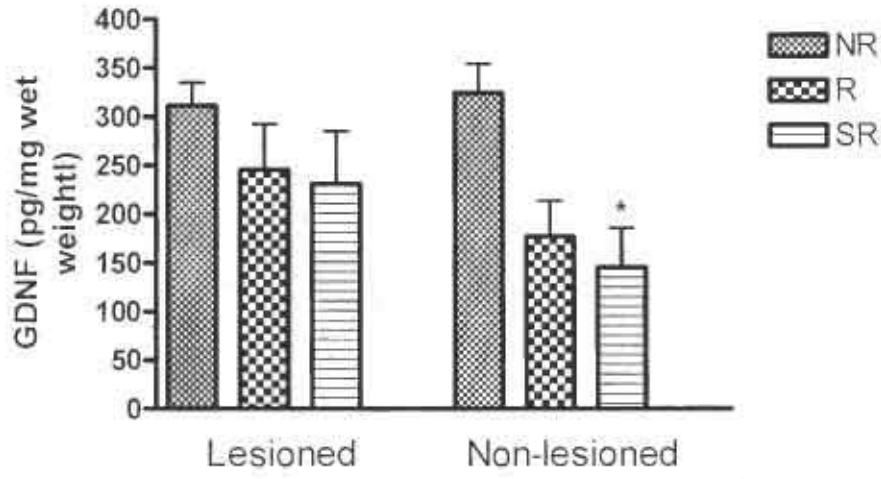


Figure 3.4.5.2 GDNF concentration in the lesioned and non-lesioned substantia nigra of the NR (n=14), R (n=11) and SR (n=11) rats. *(NR (non-lesioned) vs SR (non-lesioned), $p<0.05$). Data reported in Table 3.4.5.1.

3.4.5.3 GDNF concentration in the VTA

There was no significant difference between the GDNF concentration in the VTA of lesioned and non-lesioned hemispheres of the NR, R and SR rats *(Table 3.4.5.1, Figure 3.4.5.3)*.

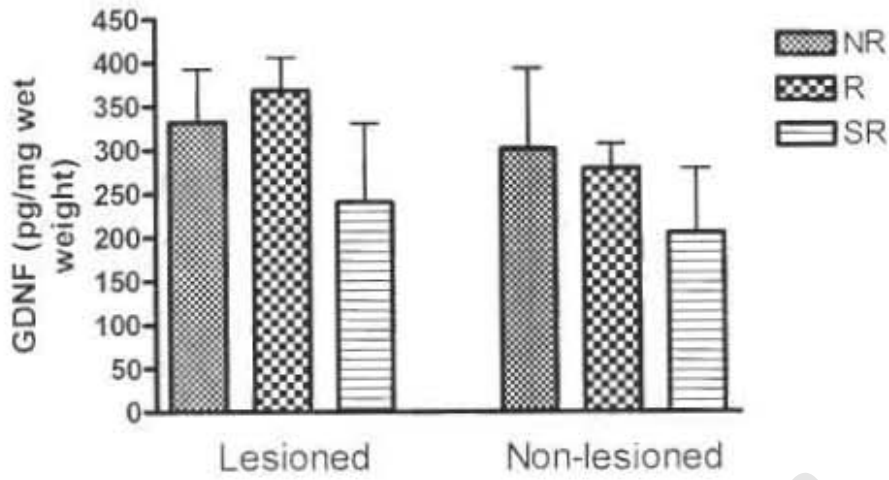


Figure 3.4.5.3 GDNF concentration in the lesioned and non-lesioned VTA of the NR (n=14), R (n=11) and SR (n=11) rats. Data reported in Table 3.4.5.1.

3.5 DISCUSSION

The results of this study suggest that rats that had free access to running wheels (R) during the experiment had increased basal ACTH and corticosterone levels. However corticosterone levels were increased in both groups (R and SR) that had access to free running wheels. The GDNF concentration in the substantia nigra of the non-lesioned hemisphere of rats that were stressed and had access to running wheels was significantly less than the GDNF concentration in the substantia nigra of the non-lesioned hemisphere of rats without running wheels.

In the present study, the mean daily distance run by the non-stressed rats (NSR) and the rats that were stressed increased steadily from day 1 until the day of 6-OHDA infusion 7 days later. Following 6-OHDA lesion, the rats in the stressed group attained pre-lesion running distances more quickly than the rats that were not stressed. The mean daily running revolutions made by the rats in the stressed group were almost twice as much as the mean daily running revolutions made by the rats that were not stressed. Basal ACTH levels and corticosterone levels of the non-stressed rats with access to running wheels were significantly greater than the basal ACTH levels and corticosterone levels of the non-stressed rats without running wheels. In normal rats, physiological stressors activate the release of CRF in the hypothalamus which in turn acts on the anterior pituitary facilitating the release of ACTH into the circulation which results in the activation of the adrenal glands to release corticosterone *(Section 1.6)*. One of the physiological functions of corticosterone secretion is to stimulate gluconeogenesis and the mobilisation of amino acids and fatty acids for energy production *(Tharp 1975)*. *Ploughman et al (2005)* have shown that voluntary exercise results in an increase in basal corticosterone concentration in rats. This increase in basal corticosterone levels is thought to be due to an increase in energy requirements as there was a positive correlation between an increase in running distance and circulating plasma corticosterone *(Ploughman et al 2005)*. In the stressed rats with access to running wheels, the exposure to various stressors for 14 days after 6-OHDA infusion did not seem to increase ACTH

levels as there was no significant difference between the basal ACTH levels of the stressed rats with access to running running wheels and the non-stressed rats without running wheels. However the basal corticosterone levels of the stressed rats with attached running wheels was significantly different from the basal corticosterone levels of the rats in cages with immobilised running wheels. This might suggest that the stressors that the rats in the stressed group were exposed to in the two weeks following 6-OHDA resulted in an increase in circulating corticosterone which activated the HPA axis negative feedback loop thus decreasing the circulating ACTH levels. It has been suggested that changes in glucocorticoid response to training appear to be produced by adaptations of the HPA axis which reduces the ACTH release in response to stress *(Tharp 1975)*. Studies have also shown that moderate to exhaustive exercise progressively increase circulating glucocorticoids *(Tharp 1975, Ploughman et al 2005)*. In the present study, when the stressed rats with running wheels attached were deprived of food for 24 h, they exercised to exhaustion as shown by the dramatic decline in running revolutions on subsequent days. Therefore it seems that in exercise an increase in corticosterone concentration can occur in the absence of a corresponding increase in plasma ACTH concentration. *Ploughman et al (2005)* have shown that the corticosterone concentration in rats exposed to voluntary wheel running was significantly less than the corticosterone concentration of rats exposed to a 30 min run or a 60 min walk on a treadmill *(Ploughman et al 2005)*. This might suggest that the increase in circulating corticosterone levels in treadmill running is also due to the stress of forced exercise. Therefore exposure to stress during exercise exacerbates the corticosterone response however in our stress model, there was no significant difference between the basal corticosterone concentrations of the non-stressed and stressed rats both with access to running wheels. Plasma ACTH and corticosterone levels were elevated in 6-OHDA lesioned runners suggesting that the discomfort caused by exercising the impaired limb was stressful perhaps increasing demand on energy reserves increasing ACTH and corticosterone secretion to stimulate gluconeogenesis.

The adverse effects of corticosterone in the brain include increasing the vulnerability of neurons to injury *(Schaff et al 1998).* One of the mechanisms with which corticosterone increases neuronal vulnerability to injury is by down regulating neurotrophic factor gene expression in the affected brain area *(Section 1.5.3, Schaff et al 1998).* It therefore stands to reason that an increase in circulating corticosterone would result in a decrease in GDNF concentration in non-stressed rats in cages with running wheels and in rats that were stressed and had access to running wheels. However in the present study, there was no significant difference between the GDNF concentration in the striatum and VTA of all the rats. GDNF concentration was significantly lower only in the substantia nigra of the non-lesioned hemisphere of exercised rats that were stressed when compared to the GDNF concentration in the substantia nigra of the non-lesioned hemisphere of the non-stressed rats without running wheels.

In a study that looked at the effects of exercise on neurotrophic factor levels, it was found that there was a positive relationship between the distance run and hippocampal levels of BDNF, synapsin-1 and pCREB *(Ploughman et al 2005).* (BDNF, synapsin-1 and pCREB have been discussed in Section 1.5.1).
Short duration walks increased hippocampal BDNF levels more than treadmill running of 2 km or more *(Ploughman et al 2005).* In the present study the stressed rats with access to running wheels ran consistently more than 2 km a day. In a previous study done in our lab, using the same experiment protocol as in the present study, *Howells et al (2005)* found that there was no significant difference between the apomorphine-induced turns made by the stressed rats with access to running wheels and the non-stressed rats in cages with immobilised wheels following 6-OHDA lesion. However the apomorphine-induced turns made by non-stressed rats with access to running wheels were significantly less than the apomorphine-induced turns made by the stressed rats with access to running wheels and the stressed rats without running wheels. As apomorphine induced turns are produced by striatal dopamine destruction greater than 70% of

the non-lesioned hemisphere *(Hudson et al 1993)*, this suggests that stress cancelled the beneficial effects of exercise. In Chapter 2, we found that exercise provided neuroprotection to dopamine neurons of non-stressed rats but neuroprotection was absent in rats that were in cages with immobilised running wheels. However in the present study there was no significant difference between the GDNF concentration in the striatum and VTA of all the rats. *Cohen et al (2003)* observed that following forced exercise of the injured limb, there was a significant increase in the striatal GDNF levels of the lesioned hemisphere that peaked 3 days post lesion and returned to normal levels 7 days after a unilateral 6-OHDA infusion. In the present study, the rats were sacrificed 14 days after 6-OHDA infusion suggesting the small but significant GDNF increases associated with exercise following neuronal injury could not be detected. In the present study, there was a significant decrease in the substanta nigra GDNF concentration in the stressed rats that had access to running wheels when compared to the substantia nigra GDNF concentration in the non-stressed rats without running wheels. However it must be noted that there were higher basal corticosterone levels in the rats with access to running wheels. The decrease in GDNF in the stressed rats with access to running wheels could be due to very high concentrations of corticosterone levels that might have occurred in response to the stressors, especially being present during exercise to exhaustion following the 24 h food deprivation. *Howells et al (2005)* found dopamine destruction in the substantia nigra of the non-stressed rats with running wheels tended to be 4% and 14% lower than dopamine destruction in the stressed rats with running wheels and the non-stressed rats without running wheels, respectively. This suggests that the dopamine destruction in the substantia nigra of stressed rats with running wheels tended to be lower than the dopamine destruction in the substantia nigra of non-stressed rats without running wheels. Therefore the significant decrease in GDNF concentration in the substantia nigra of stressed rats with running wheels might have occurred after the window of maximal GDNF expression suggested by *Cohen et al (2003)*.

Taken in conjunction with *Howells et (2005)* and Chapter 2, the present findings suggest that the decrease in the neuroprotective effect of exercise in stressed rats with running wheels is due to stress-induced HPA axis activation. This is suggested by the high basal corticosterone levels in the absence of increased basal ACTH levels. However the high circulating corticosterone levels do not seem to completely inhibit GDNF expression in the critcal period following 6-OHDA infusion as the substantia nigra dopamine destruction in stressed rats with running wheels tended to be lower than substantia nigra dopamine neuron destruction in non-stressed rats without running wheels.

3.6 CONCLUSION

GNDF expression is not increased 14 days after the lesion. If GDNF provides neuroprotection in exercising rats then GDNF surges are transient and decrease soon after the infusion of neurotoxins as suggested by *Cohen et al (2003)*. This is supported by the fact that in rats that started exercising after the GDNF surge had passed, there was complete destruction of dopamine neurons in the nigrostriatal pathway *(Tillerson et al 2001). Howells et al (2005)* has also shown that following apomorphine injection into 6-OHDA infused rats, the were significantly more apomorphine-induced turns made by the stressed rats with attached running wheels than in non-stressed rats with running wheels suggesting that stress cancelled the benefical effects of exercise. An increase in basal corticosterone levels in 6-OHDA infused rats that had access to running wheels does not seem to exarcebate dopamine neuron destruction or GDNF concentration but the addition of exogenous stressors results in a decrease in GDNF concentration in the substantia nigra of stressed rats. As Parkinson's disease is a progressive neurodegenerative disease prescribing a treatment protocol that involves moderate exercise and a reduction in exposure to stress might slow down the progression of the neurodegeneration.

CHAPTER 4

The effects of uninterrupted 3 week voluntary exercise on the HPA axis and GDNF levels in the nigrostriatal pathway and the VTA of nonlesioned Sprague Dawley rats.

4.1 INTRODUCTION

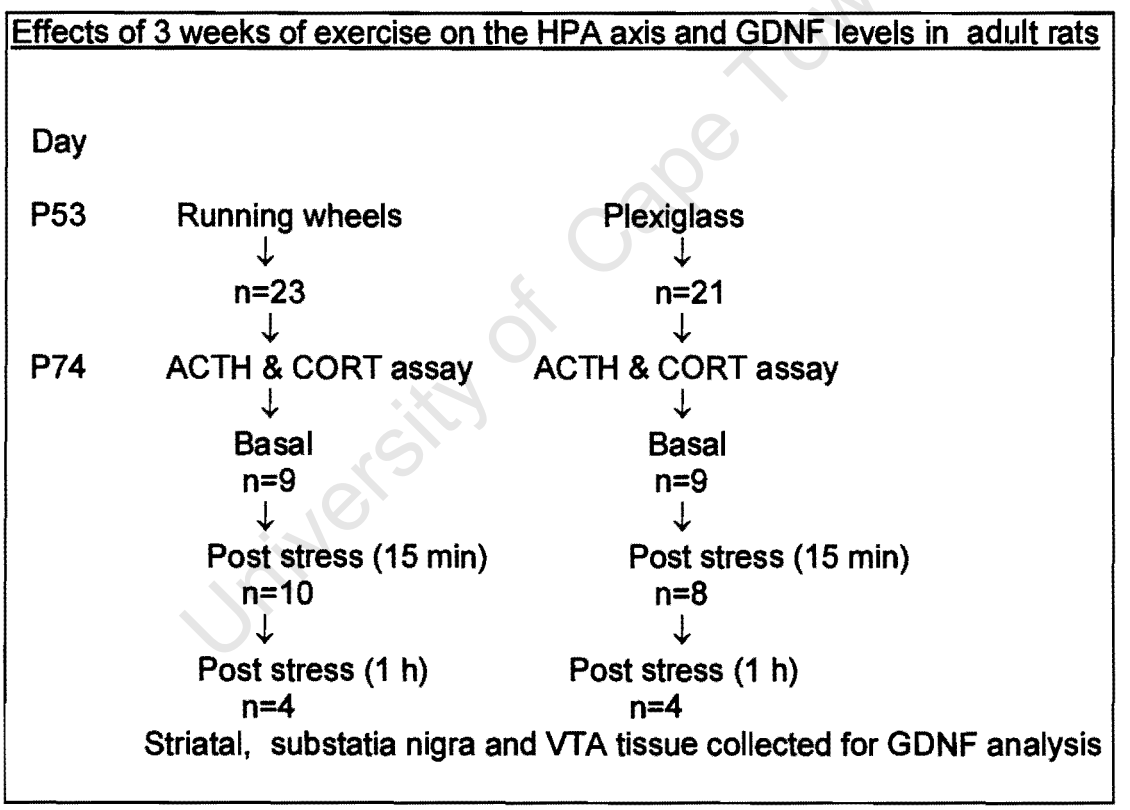
Exercise has been associated with an increase in the expression of endogenous neurotrophic factors and a growth in neuronal processes and neurogenesis *(SECTION 1.4, 1.51, 1.52).* In vitro studies have shown that the presence of GDNF facilitates an increase in the size of and length of dopamine neuron processes which form a dense network of axons and dendrites *(Lopez-Martin et al 1999).* In vivo studies have shown that dopamine neurons exposed to increased GDNF expression can withstand the toxic effects of 6-OHDA in rats *(Cohen et al 2003).* An increase in circulating glucocorticoids such as corticosterone has been shown to decrease the concentration of some neurotrophins following a toxic insult *(SECTION 1.5.3).*

If GDNF is involved in brain plasticity as shown in studies in which GDNF resulted in an increase in the size of the neurons and the size of the processes *(Lopez-Martin et al 1999),* then exercise should increase the levels of GDNF in the absence of a toxic insult to the neurons. Therefore our aim was to investigate whether prolonged voluntary exercise results in an increase in GDNF levels in the striatum, substantia nigra and VTA, and whether exercise attenuates the increase in stress hormone levels when the rats are exposed to an acute restraint stress.

4.2 MATERIALS AND METHODS

Forty-four day old Sprague Dawley rats that had been kept in a 6 am to 6pm light/dark cycle in the Departmental Animal house facility were moved to a room with a 12 h (11 am to 11pm) dark/light cycle. There were 4 rats per cage.

Table 4.2 Flow diagram of experimental protocol. Rats (R) had access to running wheels for three weeks and another group of rats (NR) were kept in plexiglass cages. P is the postnatal day on which the experiment was performed.



4.2.1 Running Experiments

Seven days later (P53), the rats were weighed and randomly divided into two groups viz. the runners and non-runners. The runners were placed in cages with running wheels and the non-runners were placed in plexiglass cages

without running wheels *(Table 4.2)*. Running revolutions were recorded daily between 10 am and 11 am (which was the hour before the rats entered the dark cycle) for three weeks. The weights of the rats were recorded on P60 and on the day the rats were subjected to acute stress.

4.2.2 Acute Stress response.

On P74, the rats were weighed and taken to the lab in which behavioural studies were performed. The rats were taken to the behavioural lab at least an hour before trunk blood was collected for basal ACTH and corticosterone determination. The remaining rats were placed in rodent holders for a 10-min period. Trunk blood was collected 15 min and 1 h post restraint.

4.2.3 Trunk blood collection.

Trunk blood collection and brain tissue dissection was performed as described in Section 3.2.4.

4.2.4 Radioimmunoassays

Plasma corticosterone levels were measured using an ImmuChem double antibody ^{125}I Corticosterone RIA kit (MP Biomedicals, LLC, Orangeburg,NY). A two-site solid phase immunoradiometric assay (IRMA) kit, EURIA-ACTH c.t. (EURO-DIAGNOSTICA,Malmö, Sweden), was used to determine the amount of ACTH in the plasma of the rats. The procedure followed was as outlined in Sections 3.2.5.1, 3.2.5.2; a standard curve was generated using standards provided in the kits. The levels of corticosterone and ACTH were calculated by interpolation of unknown values against the standard curve generated.

4.2.5 GDNF ELISA

GDNF concentration in the striatum, substantia nigra and VTA was measured as described in Section 3.2.6.

4.3 STATISTICAL ANALYSIS

Graph Pad Prism 4 was used for statistical analysis. ANOVA was used to analyse the data and when significant differences were found ($p<0.05$), post hoc comparison using Tukey's Multiple Comparison Test was performed. Results are reported as mean ± standard error of the mean (SEM).

4.4 RESULTS

4.4.1 Locomotor Activity

The mean daily revolutions increased steadily from day 1 when the rats were placed in the running wheels until day 21 three weeks later *(Table 4.4.1, Figure 4.4.1)*.

Table 4.4.1 Mean daily distance run by non-lesioned adult rats over a 3 week period.

Day	Distance travelled (m)
1	190 ± 46.2
7	1370 ± 297
13	2594 ± 624
21	3155 ± 746

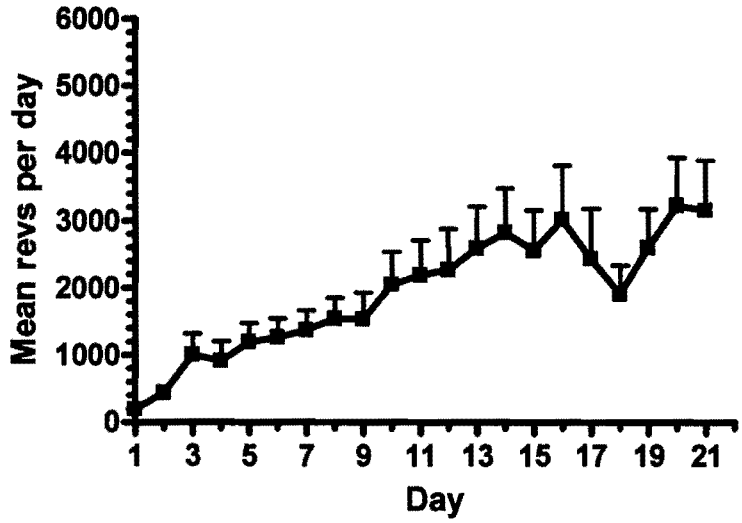


Figure 4.4.1 The mean daily distance run by non-lesioned rats (n=23) over a 3 week period. Data reported in table 4.4.1.

4.4.2 Rat Weights

On the first day of week 1 (P53) the day the rats were placed individually into their respective cages, there was no significant difference between the weights of rats with access to free running wheels (R) and the rats that were placed in plexiglass cages (NR) *(Table 4.4.2, Figure 4.4.2).*

On P60, one week after the rats were placed in their respective cages, there was no significant difference between the weights of the R and NR rats. However on P74 the NR rats weighed significantly more that the R rats *(Table 4.4.2, Figure 4.4.2).*

Table 4.4.2 Weight of non-lesioned rats with access to running wheels (R) and in plexiglass cages (NR). *(R vs NR- week 4, $p<0.001$).

Week	Weight (g)	
	R	NR
1	257 ± 5.51	257 ± 4.52
2	281 ± 4.42	285 ± 6.81
3	334 ± 5.09	369 ± 4.15*

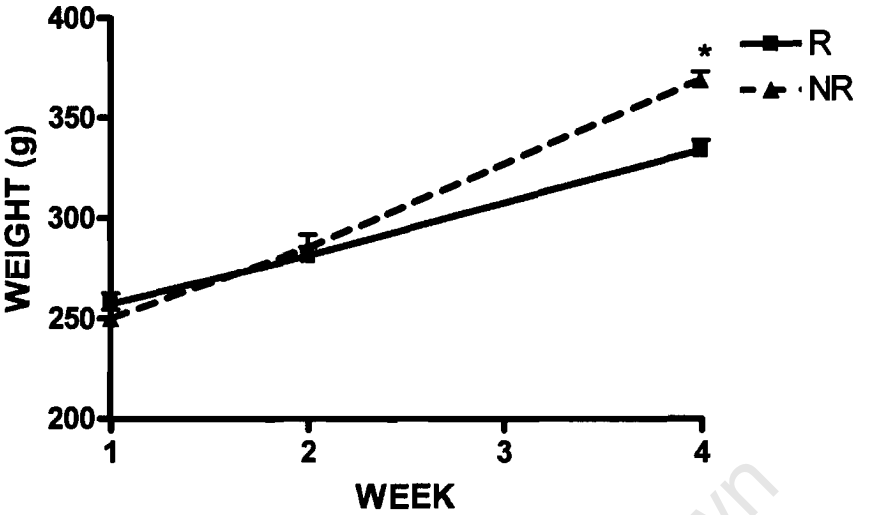


Figure 4.4.2: The weights of the rats in running wheels (R) and rats in plexiglass cages (NR). *(R vs NR- week 4, $p<0.001$). Data reported in Table 4.4.2.

4.4.3 Radioimmunoassays

4.4.3.1 ACTH assay

There was no significant difference between the plasma ACTH concentrations of the R and NR rats at all three time points (basal, 15 min and 1 h post restraint) *(Table 4.4.3.1, Figure 4.4.3.1).*

Table 4.4.3.1 Plasma ACTH concentration in 74 day old rats that had access to running wheels (R) and rats that were kept in plexiglass cages (NR) before (basal) and after restraint stress (15 min) and (1 h).

Plasma ACTH concentration (pg/ml)			
NR (basal)	R (basal)	NR (15 min)	R (15 min)
57 ± 16.4	64 ± 13.3	127 ± 28.1	106 ± 27.1
NR (1 h)	R (1 h)		
101 ± 33.9	59 ± 12.4		

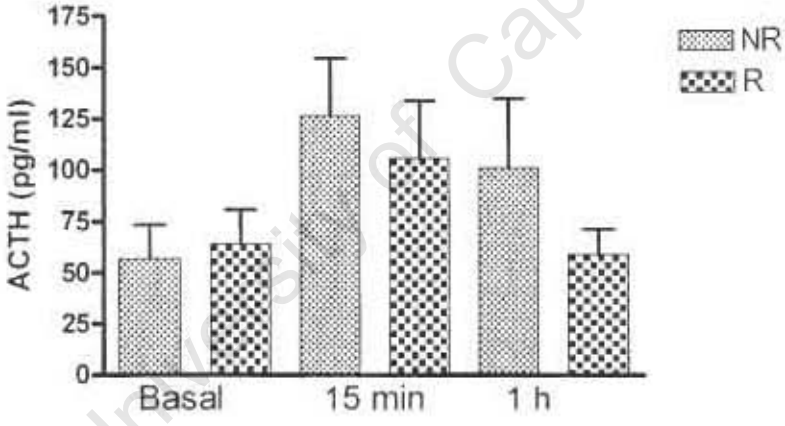


Figure 4.4.3.1 Plasma ACTH concentration in rats without running wheels before and after restraint (NR basal, n=9, NR 15 min, n=8, NR 1h, n=4). Plasma ACTH concentration in rats that were in cages with attached running wheels before and after restraint (R basal, n=9, R 15 min, n=10 and R 1 h, n=4). Data reported in Table 4.4.3.1.

4.4.3.2 Corticosterone assay.

There was no significant difference between the basal plasma corticosterone concentrations of the R and NR rats *(Table 4.4.3.2, Figure 4.4.3.2)*. There was no significant difference between the post restraint stress (15 min and 1h) corticosterone concentrations of the R and NR rats *(Table 4.4.3, Figure 4.4.3.2)*. The 15 min post restraint stress corticosterone concentrations of the NR rats were significantly higher than the basal corticosterone concentrations of the NR rats *(Table 4.4.3.2, Figure 4.4.3.2)*. There was no significant difference between the basal and post restraint stress corticosterone concentrations in the R rats and NR rats (1h) *(Table 4.4.3.2, Figure 4.4.3.2)*.

Table 4.4.3.2 Plasma corticosterone concentration in 74 day old rats that had access to running wheels (R) and rats that were kept in plexiglass cages (NR) before (basal) and after restraint stress (15 min) and (1 h). *(NR basal vs NR 15 min, $p<0.01$)

Plasma corticosterone concentration (pg/ml)			
NR (basal)	R (basal)	NR (15 min)	R (15 min)
368 ± 61.1	288 ± 79.2	671 ± 47.5*	520 ± 43.9
NR (1 h)	R (1 h)		
407 ± 40.2	360 ± 80.1		

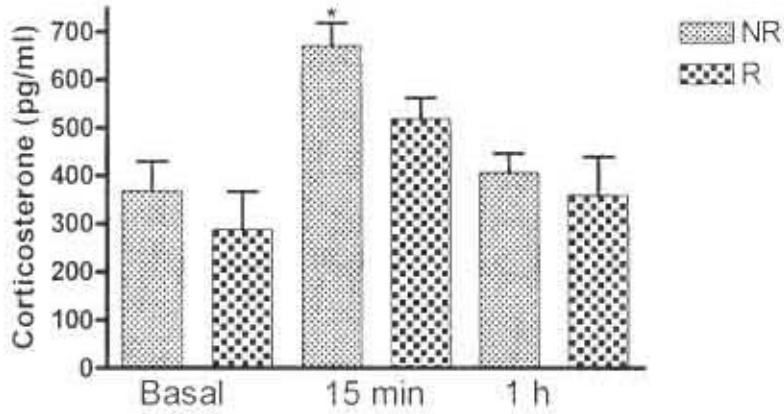


Figure 4.4.3 Plasma corticosterone concentration in rats without running wheels before and after restraint (NR basal, n=9, NR 15 min, n=8, R 1h, n=4 and NR 1h, n=4). Plasma corticosterone concentration in rats that were in cages with running wheels before and after restraint (R basal, n=9, R 15 min, n=10 and R 1h, n=4). *(NR basal vs NR 15 min, $p<0.01$). Data reported in Table 4.4.3.2.

4.4.4 GDNF concentration

In the left hemisphere, there was no significant difference between the GDNF concentration in the striatum, substantia nigra and VTA of rats with (R) or without (NR) running wheels *(Table 4.4.4, Figure 4.4.4.1)*. There was no significant difference between the striatal, substantia nigral and VTA GDNF concentration in the right hemisphere of rats that had running wheels and rats without running wheels *(Table 4.4.4, Figure 4.4.4.2)*. There was no significant difference between the mean (left plus right) GDNF concentration in rats with or without running *(Table 4.4.4, Figure 4.4.4.3)*.

Table 4.4.4 GDNF concentration in the striatum, substantia nigra and VTA of rats in cages without running wheels (NR) and rats in running wheels (R).

GDNF concentration (pg/mg wet weight)

NR

Striatum		SN		VTA	
Left	Right	Left	Right	Left	Right
34 ± 6.4	32 ± 5.5	216 ± 40.4	243 ± 29.3	319 ± 45.6	319 ± 47.1

R

Striatum		SN		VTA	
Left	Right	Left	Right	Left	Right
35 ± 6.5	28 ± 5.2	153 ± 28.1	153 ± 28.6	251 ± 47.8	240 ± 36.8

Pooled GDNF concentration (pg/mg wet weight)

NR	R
131 ± 57.0	70 ± 35.01

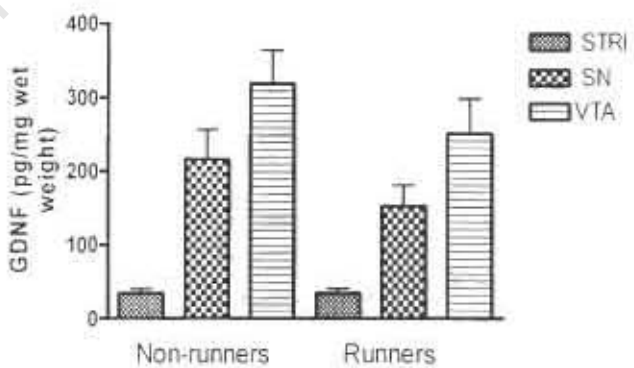


Figure 4.4.4.1 GDNF concentration in the left striatum (STRI), substantia nigra (SN) and VTA of rats without running wheels, Non-runners (n=21) and rats that had running wheels attached Runners (n=23).

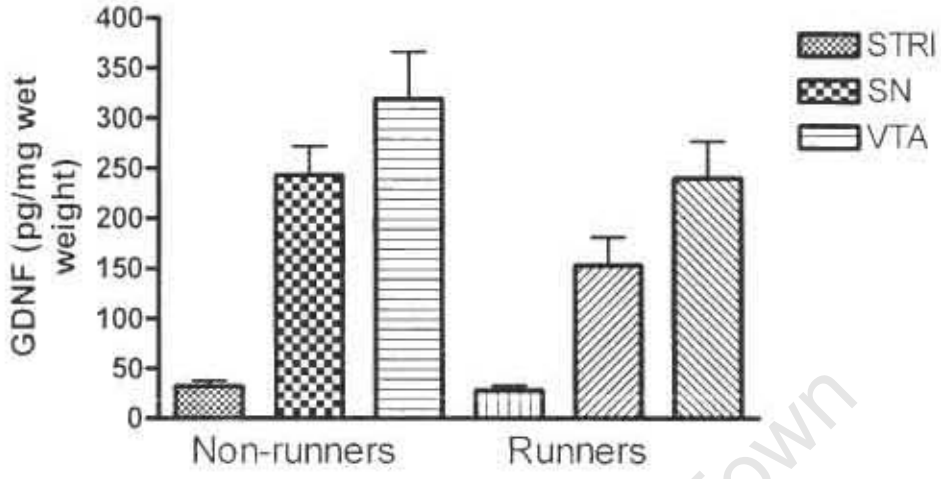


Figure 4.4.4.2 GDNF concentration in the right striatum (STRI), substantia nigra (SN) and VTA of rats without running wheels Non-runners (n=21) and rats that running wheels attached Runners (n=23).

4.5 DISCUSSION

Effects of continuous voluntary running on non-lesioned Sprague Dawley rats resulted in rats that weighed less and had similar brain GDNF concentration to rats that did not exercise. Plasma corticosterone levels were significantly increased above basal corticosterone concentrations 15 min after exposure to an acute stressor. The mean daily distance travelled by the rats increased steadily from less than 1 km per day to more than 3 km per day three weeks later. Although not statistically significant, there was a two fold increase in plasma ACTH concentration of non-exercised rats 15 min post restraint. This increase might have been large enough to activate the adrenal cortex to significantly increase corticosterone levels 15 min post restraint in rats that did not exercise. *Ploughman et al (2005)*, showed that forced or voluntary exercise of more than 30 min a day resulted in increased basal corticosterone levels. In the present study, the rats were running more than 3 km a day at the time of trunk blood collection but the basal corticosterone concentrations were not increased. However in *Ploughman et al (2005)*, the exposure to exercise was intermittent with periods of non-exposure to running wheels that lasted for as long as 4 days. One of the reasons for increased corticosterone levels was thought to be a physiological need to increase energy to maintain the exercise regimen *(Ploughman et al 2005)* however in the present study because of the continuous nature of the exercise regimen, the rats might have adapted to the stress of exercise hence exposure to an acute stress even though probably more severe than voluntary exercise did not result in significantly elevated plasma corticosterone levels.

In rat studies, an increase in GDNF levels has been associated with neuroprotection following neurotoxic injury *(Tillerson et al 2001, Cohen et al 2003, Smith et al 2003)* in exercising rats. A GDNF increase and thus neuroprotection was measured by assessing the GDNF levels in the injured and uninjured hemispheres. In the present study there was no neurotoxic insult and thus both hemispheres were intact. However there was also no significant difference between rats with access to running wheels and rats that did not exercise. An explanation for this might be that in rat brains GDNF expression rapidly decreases as development proceeds *(Smith et al 2003,*

Blum et al 1995). This might suggest that GDNF concentrations increase only when there is an injury to the brain. In the previous study GDNF expression in exercising rats tended to be increased in the injured hemisphere even though not significantly different from the non-lesioned hemisphere Section 3.4.5. This might support *Cohen et al's (2003)* finding that an increase in GDNF concentration is only evident immediately following injury in adult rat brains. The GDNF concentration in the non-lesioned hemispheres of these rats was not different from the GDNF concentration in the corresponding hemispheres of the rats in the present study.

4.6 CONCLUSION

Studies have shown that GDNF expression is maximal early in life when the neuronal circuitry is still forming *(Stromberg et al 1993)*. In vitro studies have shown that GDNF expression is associated with an increase in dopamine neuron size and in the number of axonal and dendritic process *(Lopes-Martin et al 1999)* suggesting that the sturdiness of the neurons can be crucial in withstanding the toxic effects of 6-OHDA. However GDNF expression rapidly decreases as development proceeds *(Stromberg et al 1993)* and increases in GDNF expression in adult rats occur when there is injury to the neurons *(Naveilhan et al 1997)*. *Cohen et al (2003)*, has shown that GDNF expression is exacerbated in the lesioned hemisphere of exercising rats. In Chapters 2 and 3, we found that in the absence of exogenous stressors, exercise provides neuroprotection following 6-OHDA infusion in the MFB. In the present study we found that ACTH and corticosterone levels in plasma were not significantly increased after acute restraint stress. However the rats in the present study were not lesioned and hence would not have been as stressed as the rats discussed in Section 3 during exercise. It has also been suggested that Sprague Dawley rats are diurnally inactive *(Schallert et al 2000)* suggesting that as the rats were taken out of their cages with attached running wheels during their light cycle, the need for energy mobilisation by increasing corticosterone levels would have been low. The absence of a corticosterone response to restraint stress in exercised rats might suggest that the neuronal circuitry of the HPA axis adapts to the stress of exercise by

marginal desensitisation of the CRF receptors in the pituitary gland thus limiting the secretion of ACTH during transient increases in stress. The absence of an increased GDNF expression in rats with access to running wheels suggests that GDNF changes after the perinatal period only occur in the presence of brain trauma. Increase in GDNF expression has also been shown to be transient even in the presence of brain injury suggesting that an increase in GDNF expression is unlikely to be seen 3 weeks after exercise was started. Therefore the beneficial effect of exercise in non-lesioned rats seems to be the increased threshold for ACTH and thus corticosterone release. As GDNF has been shown to be negatively correlated to corticosterone levels, exercise pre and post lesion might be beneficial in negating corticosterone surges during and after 6-OHDA lesion.

CHAPTER 5

Development of a mild prenatal stress rat model to study long term effects on neural function and survival.

5.1 INTRODUCTION

Stress during gestation results in brain malformation that has effects that are present in the adult offspring *(Section1.7. 4)*. Prenatal stress models commonly used include chronic models that are present throughout the duration of gestation or acute models that expose the dam to stressors during the last week of gestation *(Section 1.7)*. The severity of these stressors can be correlated to the behavioural abnormalities that are present in adult offspring of rats that were prenatally stressed *(Section 1.7)*.

Models of gestational stress that have been used include food deprivation models (Kehoe *et al* 2001, Lesage *et al* 2002, Ježová *et al* 2002) where pregnant rats are either food deprived during the perinatal period or subjected to variable stressors including daily handling and saline injections during the last week of gestation (Ward *et al* 2000).

The aim of this study was to develop a mild stress model that did not demonstrate anxiety or weight loss in adulthood. Although the variable stressor model *(Ward et al 2000)* has been regarded as a mild stress model, the model proposed in this study does not involve physical discomfort to the rat (e.g. saline injection) a factor which might cause anxiety during pregnancy. We also investigated the effects of prenatal stress caused by food deprivation on the HPA axis of the adult offspring. This model was compared to the food deprivation models discussed in Section 1.75, by measuring corticosterone and ACTH levels at two months of age. However this food deprivation model differed from the 28 day model proposed by Lesage (Lesage *et al* 2002) in that it was of shorter duration (6 days). It also differed from the Ježová model in that food consumption

was restricted to 50 % of that consumed by the rats in the first week of gestation whereas in the Jezova model, there was 75% food deprivation (Ježová *et al* 2002).

5.2 MATERIALS AND METHODS

Forty-eight adult Sprague Dawley rats (24 female and 24 male) were housed under standard laboratory conditions with a 6 am to 6 pm light/dark (L/D) cycle and free access to food and water.

Table 5.2 Flow chart of experimental protocol for non-stressed rats (Group C), 50% food deprived rats (Group F) and rats receiving various mild stressors (Group S)

	Group C	Group F	Group S
	12 breeding pairs	6 breeding pairs	6 breeding pairs
	↓	↓	↓
*Pre	non-stressed	food deprivation	mild stressors
	↓	↓	↓
P2	18 male pups	21 male pups	20 male pups
	↓	↓	↓
P60	Behavioural tests	Behavioural tests	Behavioural tests
	↓	↓	↓
[1]P66		ACTH & CORT assay	
	↓	↓	↓
	n=6 basal	n=6 basal	n=6 basal
	n=6, 15-min post stress	n=6, 15-min post stress	n=6,15-min post stress
	n=6, 30-min post stress	n=6, 30-min post stress	n=6, 30-min post stress

*Prenatal stress protocol applied during 3rd week of gestation
[1] Adrenal glands from all the rats were dissected out and weighed.

5.3 Breeding

Female rats were grouped together (four per cage) for eight weeks so as to synchronise their ovarian cycles. They were then placed in individual cages and vaginal smears were taken daily for 4 days to monitor their oestrous cycles. A rat's ovarian cycle is normally 4-5 days long and is divided into several phases: (1) proestrus, (2) oestrus, and (3) dioestrus, a 48-hour period that is divided into dioestrus 1 and then dioestrus 2 (Marcondes *et al., 2002*). Ovulation occurs from the commencement of proestrus to the end of oestrus (Marcondes *et al 2002*).

On the day of proestrous, a male, randomly selected, was placed in a proestrus female's cage. As mentioned in the introduction, the neonatal HPA axis is functional in the last week of gestation thus our experiment required stressing the pregnant dams in the last week of gestation i.e. from gestational day 14.
To determine the exact day of conception, we watched for the presence of vaginal plugs. The males were removed from the breeding cages. This was called gestational day 0 (E 0). From E1 to E 7 daily food consumption by the rats was measured.

5.4 Prenatal stress protocol

On E 14, the female rats were divided into three groups; (1) non-stressed rats (Group C, n = 12), (2) food-deprived rats (Group F, n = 6) and (3) mild multiple stressor group which we will refer to as the mildly stressed (Group S, n = 6) rats (Table 5.2).

Non-stressed rats received food and water *ad libitum*. Food-deprived rats received 50% of the average daily food consumed by the dams during the first week of gestation. At 9 am on GND14, rats in Group S were taken to a different room where the 12 hour L/D cycle was reversed. On GND15 the reversed L/D cycle was maintained. At 9 am on GND16 the L/D cycle was changed back to the

original 6am to 6pm cycle. On GND17, food was removed from the cages for a period of 24 hours. On GND18, the rats received multiple stressors: they were placed in clean cages for a period of 5 min and then returned to their home cage for 5 min. Next, they were handled for 5 min and then placed in a cage in which the floor was covered with wire mesh for 5 min after which they were returned to their home cages. On GND19, Group S rats were returned to the room with Group C and F cages and all the rats received food and water *ad libitum*.

5.5 Postnatal handling

On postnatal day 2 (P2), the pups were sexed and females were culled leaving only males. In order to ensure that we looked only at the effects of prenatal stress, the dams in the stressed groups (50% food deprivation and mildly stressed) were removed from the study. Therefore the pups of the dams from groups F & S were cross-fostered onto the dams in group C. The pups from Group C were cross-fostered onto a different control dam to ensure an appropriate control. Only pups from the same litter were "cross-fostered" onto a dam. Fifty-nine pups were cross-fostered i.e. Group C pups n =18, Group F pups n =21 and Group S pups n =20.

These dams and "cross-fostered" pups were housed under normal Animal house conditions. Litters were kept with foster mothers until weaning (P21), after which the pups were housed 2 per cage and received food and water *ad libitum.*

5.6 Behavioural tests

5.6.1 Elevated plus maze

At P60, all experimental offspring were tested in the elevated plus maze. The elevated plus maze apparatus consists of two open arms and two closed arms and is used to estimate the level of anxiety of a rat (Daniels *et al* 2004). Daniels *et al* showed that the more anxious a rat is, the more time it will spend in the closed arm of the maze (Daniels *et al* 2004). Each rat spent 5 min in the elevated plus maze; the number of entries into each arm was recorded by a video camera placed strategically above the elevated plus maze to cover every angle of the maze. Noldus software was used to determine the amount of time spent in the open and closed arms. The 5-min time interval is considered to be an optimal amount of time for the elevated plus maze as fatigue becomes a confounding factor in longer tests (Daniels *et al* 2004). Following the tests in the elevated plus maze, the rats were allowed to recover for 2 hrs in their cages before being tested in the open field box.

5.6.2 Open field

The open field apparatus is used to measure activity of the rats in a novel environment *(Colorado et al 2006)* which includes locomotor activity (line crossing), exploration (rearing) and fear and anxiety (centre square entries) *(McFadyen-Leussis et al 2004)*. The open field activity box measured (1 m X1 m X 0.5 m) with charcoal grey fiberglass flooring and the four lateral sides painted cream. The inner zone measured (0.7 m X 0.7 m). Each rat was tested for a period of 5 min. To aide with the analysis, a video camera was used to record behavioral activity. Following the tests, the rats were returned to the depatmental animal facility.

5.6.3 Stress response

At P66, 6 rats from each group were decapitated and trunk blood was collected for basal corticosterone and ACTH determination. Adrenal glands were dissected and weighed.
The remaining rats were placed in rodent holders for a 10-min period. Trunk blood was collected either 15 min (n = 6 from each group) or 30 min (n = 6) post restraint. Adrenal glands were collected from all the rats.

5.6.4 Radioimmunoassays

Plasma corticosterone levels were measured using an ImmuChem double antibody ^{125}I Corticosterone RIA kit (MP Biomedicals, LLC, Orangeburg,NY).
A two-site solid phase immunoradiometric assay (IRMA) kit, EURIA-ACTH c.t. (EURO-DIAGNOSTICA,Malmö, Sweden), was used to determine the amount of adrenocorticotropic hormone (ACTH) in the plasma of the rats. The procedure followed was as outlined in the kit manuals and described in Section 3.2.5.1; a standard curve was generated by using standards provided in the kits and then the levels of corticosterone and ACTH were calculated by interpolation of unknown values against the standard curve generated.

5.7 STATISTICAL ANALYSIS.

Statistica 7 (Statsoft Inc, Oklahoma, USA), was used to perform the Levene test of homogeneity of variance, analysis of variance (ANOVA) and Tukey's post hoc test on the data. Results are reported as mean ± standard error of the mean (SEM).

5.8 RESULTS

5.8.1 Elevated plus maze

There was no significant difference between the non-stressed, mildly stressed and food-deprived offspring in the amount of time the rats spent in the open or the closed arms of the elevated plus maze *(Table 5.8.1, Fig 5.8.1).*

Table 5.8.1 Amount of time spent by the rats in the open and closed arms of the elevated plus maze.

Time in seconds		
Open arm		
Non-stressed	Mildly stressed	Food deprived
65 ± 8.65	67 ± 6.32	52 ± 9.62
Closed arm		
Non-stressed	Mildly stressed	Food deprived
146 ± 8.41	158 ± 8.28	165 ± 8.58

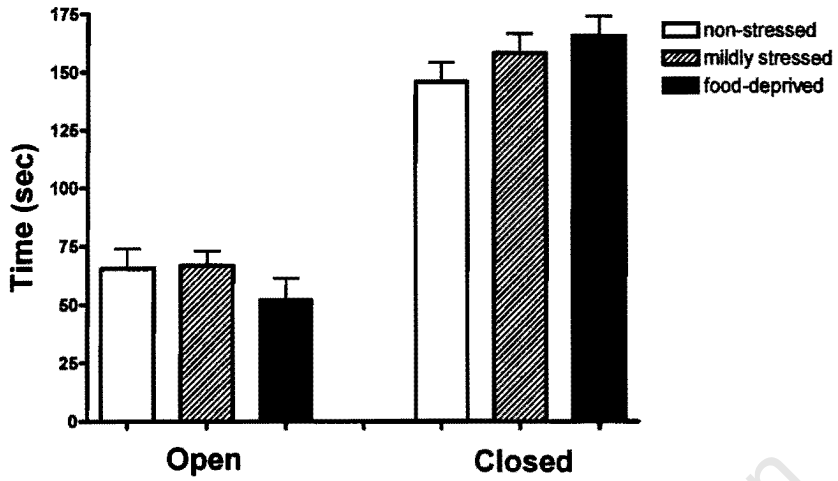


Figure 5.8.1 Time spent in open and closed arms of the elevated plus maze. Non-stressed rats (n=18) refers to 60-day old offspring of dams that received food and water *ad libidum,* "mildly stressed" (n=19) refers to 60-day-old offspring of dams that were subjected to the mild stress protocol and "food-deprived" (n=20) refers to 60-day-old offspring of dams that were subjected to 50% food deprivation during the 3rd week of gestation. Data reported in Table 5.8.1.

5.8.2 Open field test

The total distance run by the mildly stressed rats in the open field was significantly less than the total distance run by the non-stressed rats *(Table 5.8.2, Figure 5.8.2).* No significant difference was found between the total distance run by the non-stressed rats and the food-deprived rats *(Table 5.8.2, Figure 5.8.2).*

Table 5.8.2 Mean total distance covered by the rats in the open field.

* (non-stressed vs mildly stressed, $p<0.05$).

Total Distance (mm)		
Non-stressed	*Mildly stressed	Food deprived
3926 ± 558	3349 ± 610	3672 ± 150

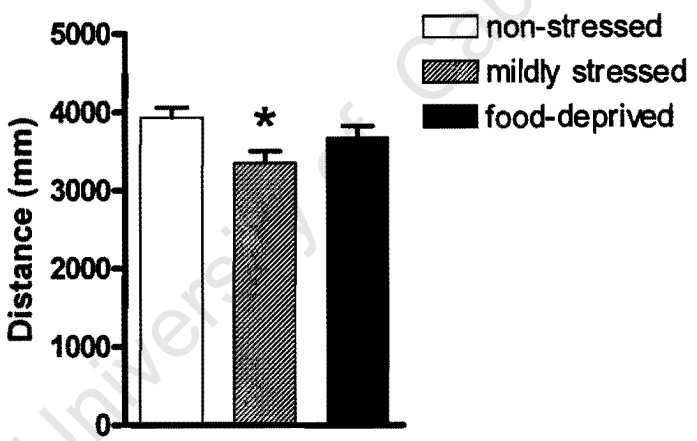


Figure 5.8.2 Total distance travelled by the non-stressed (n=18), mildly stressed (n=17) and food deprived (n=17) rats during a 5-min interval in the open field. * (non-stressed vs mildly stressed, $p<0.05$). Data reported in Table 5.8.2.

5.8.3 Adrenal weight.

No significant difference was found between the weights of the adrenal glands (in grams) of the offspring of non-stressed, mildly stressed and food-deprived dams *(Table 5.8.3, Fig 5.8.3).*

Table 5.8.3 The average weight of the adrenal glands in the non-stressed, mildly stressed and food-deprived rats.

	Weight (g)	
Non-stressed	Mildly stressed	Food-deprived
0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01

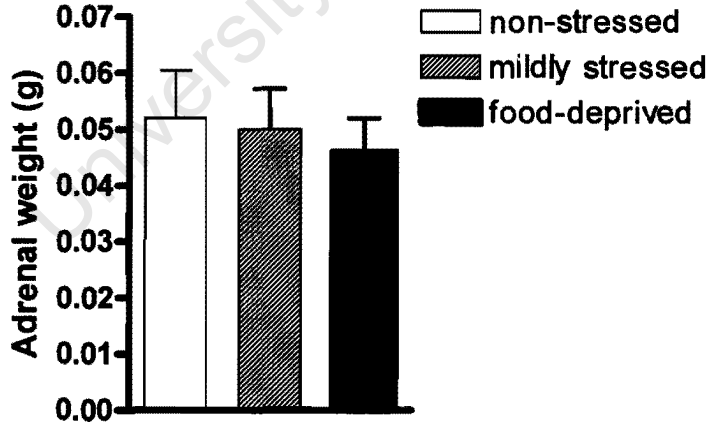


Figure 5.8.3 Adrenal weights of 66-day-old offspring of non-stressed (n=18), mildly stressed (n=18) and food-deprived (n=18) dams. Data reported in Table 5.8.3

5.8.4 Corticosterone

At 15 min and 30 min post restraint, corticosterone levels of all rats were significantly raised when compared to baseline values within the same group *(Table 5.8.4, Fig 5.8. 4).*

Non-stressed rats at 0-min, 15-min and 30-min did not differ significantly from mildly stressed and food-deprived rats at the same time intervals *(Table 5.8.4, Fig 5.8. 4).*

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Table 5.8.4 The plasma corticosterone concentration in non-stressed, mildly stressed and food-deprived rats before (0 min) and after restraint (15 min and 30 min). *(Significantly different from 0 min level, $P < 0.05$).

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Corticosterone concentration (pg/ml)		
Non-stressed (0 min)	Mildly stressed (0 min)	Food-deprived (0 min)
65 ± 27.7	116 ± 33.7	74 ± 19.2
Non-stressed (15 min)	Mildly stressed (15 min)	Food-deprived (15 min)
394 ± 38.7*	415 ± 45.7*	401 ± 27.6*
Non-stressed (30 min)	Mildly stressed (30 min)	Food-deprived (30 min)
325 ± 52.5*	317 ± 57.1*	352 ± 38.7*

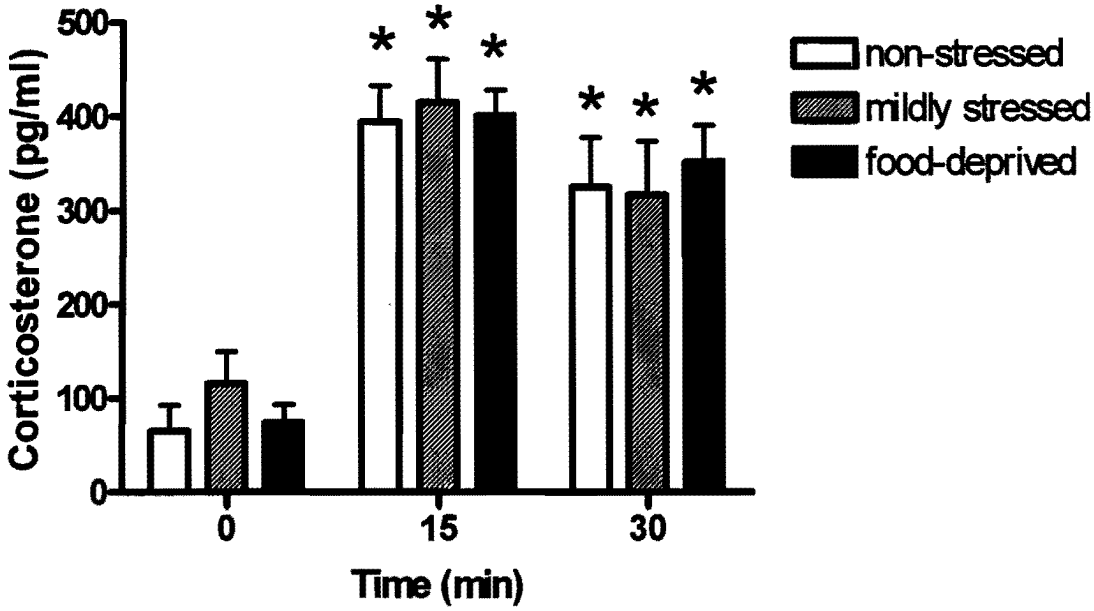


Figure 5.8.4 Plasma corticosterone levels of adult offspring of non-stressed (n=18), mildly stressed (n=18) and food-deprived (n=18) dams prior to 10-min restraint stress (0 min), 15 and 30 min post restraint stress. *Significantly different from 0 min level, $P < 0.05$.

5.8.5 ACTH

There was no significant difference between the plasma ACTH concentration of the three groups of rats prior to acute restraint stress, baseline values were very similar *(Table 5.8.5, Figure 5.8. 5)*. No significant difference was found in the plasma ACTH levels of the non-stressed, mildly stressed and food-deprived rats 15 min post restraint however following correction for multiple comparison, only the plasma ACTH concentration of the non-stressed rats was significantly elevated when compared to baseline concentrations *(Table 5.8.5, Figure 5.8. 5)*. No significant difference was found between the plasma ACTH levels of the three groups of rats 30 min post restraint *(Table 5.8.5, Figure 5.8.5)*.

Table 5.8.5 The plasma ACTH concentration in non-stressed, mildly stressed and food deprived rats before (0 min) and after restraint (15 min and 30 min). *(non-stressed (0 min) vs non-stressed (15 min), $P < 0.05$).

ACTH concentration (pg/ml)		
Non-stressed (0 min)	Mildly stressed (0 min)	Food deprived (0 min)
46 ± 8.40	46 ± 9.90	48 ± 7.40
Non-stressed (15 min)	Mildly stressed (15 min)	Food deprived (15 min)
125 ± 13.5*	109 ± 13.3	109 ± 7.80
Non-stressed (30 min)	Mildly stressed (30 min)	Food deprived (30 min)
97 ± 11.5	114 ± 31.1	95 ± 23.8

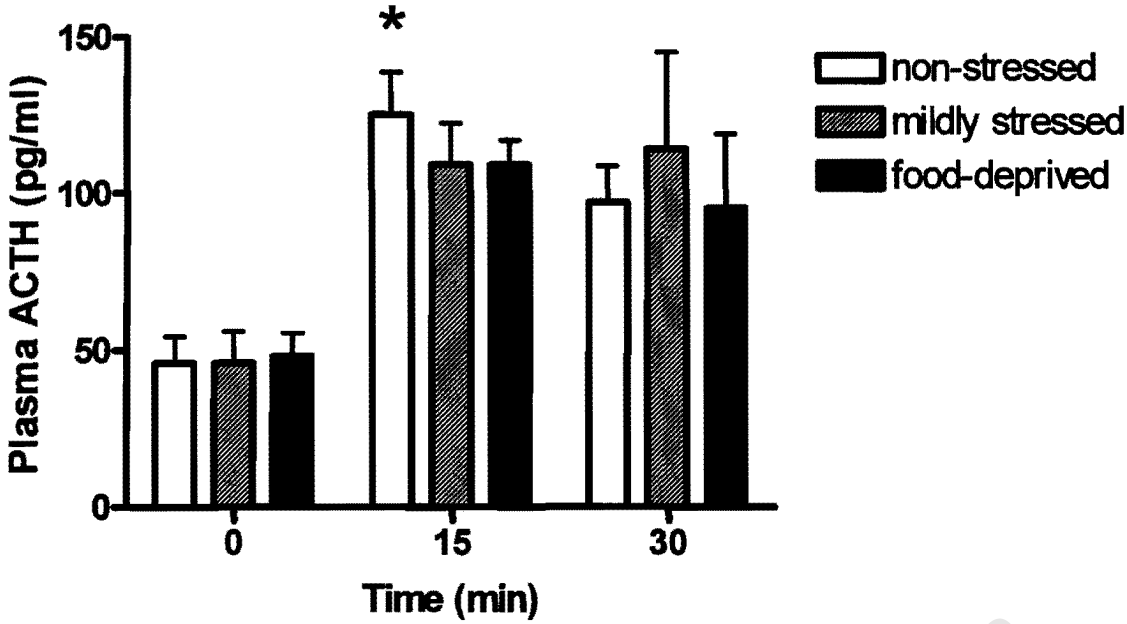


Figure 5.8.5 The plasma ACTH concentration in non-stressed (n=18), mildly stressed (n=18) and food-deprived (n=18) rats before (0 min) and after restraint (15 min and 30 min). *(non-stressed (0 min) vs non-stressed (15 min), $P < 0.05$).

5.9 DISCUSSION

It has been demonstrated in rat models that chronic or severe stress can cause disruption to the HPA axis and thus the body's physiology. Our aim was to determine whether long term changes in behaviour and endocrine function occurred in rat pups when the pregnant dams were mildly stressed during the last week of gestation. To carry out this aim, we began by establishing three groups; a non-stressed control group of dams that received food and water *ad libitum* from the first day of gestation and two groups of dams that were subjected to stress; (1) a mildly stressed group that received multiple stressors from the 14th day of gestation until the rats were ready to litter and (2) a food-deprived group that received 50% of the average daily food consumed by all the dams during the first week of gestation.

Behavioural tests were performed on adult offspring to determine the presence of any long-term effects of prenatal stress. The first test, activity in the elevated plus maze, has been used as a test of the anxiety levels of rats (Daniels *et al* 2004). Prenatal models of stress (Darnaudéry *et al* 2004) as well as postnatally stressed rats (Daniels *et al* 2004) showed a tendency to spend more time in the closed arms than in the open arms of the elevated plus maze.

The rats in the present study did not demonstrate anxious behaviour. There was no significant difference between the amount of time that rats in the three groups spent in the closed or the open arms of the elevated plus maze. The absence of this type of behaviour in the prenatally stressed rats suggests that these rats were not anxious. This contrasts with the behaviour of other prenatal rat stress models when placed in this novel environment (Darnaudéry *et al* 2004). Early life stressors have been shown to alter the development of the HPA axis (Lesage *et al* 2002) and this may affect the ability of animals to explore a novel environment (Sternberg *et al* 2003). The absence of anxiety in the present study may suggest

that the mild stress applied prenatally to rats was not as severe as that applied in other studies and therefore less interference with development of the neural circuitry that controls HPA axis function.

Decreased motor activity in the open field has been associated with anxiety or fear (McFadyen-Leussis *et al* 2004). Previous studies showed that prenatally stressed rats were less inclined to explore the open field (Kofman 2002, Nishio *et al* 2001). However in studies in which the prenatally stressed rats were cross-fostered onto non-stressed dams (Fujioka *et al* 2001), there were no differences between the locomotor scores of stressed and non-stressed rats. In our study, we found no significant difference between the amount of time spent in the outer zone or inner zone of the open field by non-stressed and prenatally stressed rats. As expected, all rats spent more time in the outer zone than in the inner zone of the open field (Fujioka *et al* 2001). Also, no significant difference was found between the total distance travelled by the non-stressed and the food-deprived rats and between the food-deprived rats and the mildly stressed group. However, a significant difference was observed between the total distance travelled by the mildly stressed rats and the non stressed rats with the mildly stressed rats covering a lesser distance than the non-stressed rats. The decreased locomotor activity displayed by the mildly stressed rats suggests a small degree of anxiety and agrees with the results of other prenatally stressed rat models *(Kofman 2002, Nishio et al 2001).*

Prenatal stress can also cause changes in the size of the adrenal glands. The mild prenatal stress model of *Ward et al (2000)* showed adrenal hypertrophy whereas food-deprived models displayed either adrenal hypotrophy (Lesage *et al 2002)* or normal adrenal glands (Ježová *et al* 2002). Chronic stimulation of the adrenal glands by high levels of circulating ACTH was suggested to be the cause of the adrenal hypertrophy in the mild stress model (Ward *et al* 2000). In the present study, the rats were exposed to stressors only in the last week of gestation (6 days) and the dams did not receive saline injections. Rats were also

cross-fostered to dams that had not been exposed to stress during gestation which differs from the model proposed by *Ward et al (2000)*. The absence of high levels of circulating ACTH in the present study could account for the absence of adrenal hypertrophy.

In the Lesage model of food deprivation, rats were 50% food deprived from the last week of gestation through to weaning. This meant that the dams were food deprived for 28 days. The small size of the adrenal glands in this model was possibly the result of chronic undernutrition in the first weeks of life because at 4 months these rats had lower body mass than controls (Lesage *et al* 2002). In the Ježová model the rats received only 25% of the daily consumption of controls but the food deprivation lasted for only 7 days, the last week of gestation (Ježová *et al* 2002). Similarly in the present study the rats were 50% food deprived for 6 days, and no significant differences appeared between the food-deprived rats and the non-stressed rats. The shorter duration of the food deprivation in our model and the Ježová model when compared to the Lesage model could be the reason for the absence of adrenal hypotrophy in these two studies.

In the 50% food deprivation model proposed by Lesage, following a 30-min restraint stress, the plasma corticosterone levels were significantly higher than baseline levels but were not significantly different from controls nor were basal levels appreciably different (Lesage *et al* 2002). In the 75% food-deprivation model (Ježová *et al* 2002) additional stressing of adult rats consisted of opening the cage and handling the rats gently for 1 min. No significant difference was observed in the basal, 15 min and 30 min post stress plasma corticosterone levels of stressed and control rats (Ježová *et al* 2002) which is in agreement with the present study.

There was no significant difference between the baseline corticosterone levels of the food-deprived rats and non-stressed rats. Also no significant difference was found in the corticosterone levels at 15 and 30-min post restraint stress which

was similar to the Ježová model. Acute restraint stress produced the expected rise from baseline values in the corticosterone levels after 15 min and 30 min post restraint (Lesage *et al* 2002). In agreement with published data (Lesage *et al* 2002), plasma corticosterone levels had not returned to baseline levels after 30 min.

In the variable stressor model of *Ward et al. (2000)* where prenatal stress included a painful stimulus, basal corticosterone levels were significantly elevated compared to control rats. In the present study, no difference in basal corticosterone levels was observed between the mildly stressed rats and the non-stressed rats. This is in agreement with the prenatal stress model of *Smith et al (2004)* in which pregnant dams were restrained for 1 h per day from gestational day 10 to 20 *(Smith et al 2004). In* the *Smith et al.* model, there was no difference between the basal plasma corticosterone levels of prenatally stressed and non-stressed adult offspring. Following a 20-min acute restraint stress of adult offspring, corticosterone levels of prenatally stressed rats were significantly higher than non-stressed rats (Smith *et al* 2004). Variations of the *Smith et a. (2004)* model of prenatal restraint stress where the rats were stressed for 30 min three times a day between GND 15 and 19 (Szuran *et al* 2000) produced similar basal corticosterone levels in adult offspring as in the present study and the *Smith et al* model. This seems to suggest that basal corticosterone levels in adult offspring of rats that were prenatally stressed are generally similar to those of non-stressed rats (Smith *et al* 2004, Szuran *et al 2000).* However in prenatal stress models that involve a painful stimulus, the corticosterone levels appear to be perpetually elevated *(Ward et al 2000).*

In the 50% food deprivation model of *Lesage et al (2002),* there was no significant difference between the basal ACTH levels of the stressed and control rats. Following 30-min restraint, a significant increase occurred in the plasma ACTH of both the prenatally stressed rats and controls when compared to basal levels. However, the ACTH levels of prenatally stressed rats returned to baseline

values more quickly than those of the non-stressed rats. In the Ježová model, there was no difference between the baseline ACTH values of the stressed and non-stressed groups but following acute restraint stress, the ACTH levels of prenatally stressed rats were significantly higher than controls (Ježová *et al 2002).* In the present study, no significant difference was observed between baseline ACTH levels of the food-deprived, mildly stressed and non-stressed rats. A significant increase was shown in the plasma ACTH levels of the non-stressed rats 15 min post restraint however after correction for multiple comparisons, plasma ACTH levels 15 min after 10-min restraint was not significantly different from baseline values. This decrease in the ACTH response to stress has been demonstrated in other models of early life stress (Daniels *et al 2004).* Daniels *et al.* postulated that this response is due to desensitization of the CRF receptors leading to low ACTH release by the pituitary gland in response to a stressor *(Daniels et al 2004).* No significant difference in the ACTH levels was observed at 30 min post restraint stress confirming that the prenatally stressed rat models developed in the present study represent a milder form of prenatal stress than currently available models.

5.10 CONCLUSION

The 50% food deprivation model proposed in this study was of acute duration (6 days) when compared to the 50% food deprivation proposed in the Lesage model. The former did not produce any differences in the size of the adrenal glands, plasma corticosterone levels or the ACTH response to restraint stress. The duration of the early life stressor plays a role in the rat's response to stress since the 75% food deprivation model (Ježová *et al.,* 2002) was of similar duration as the present study and yielded similar results.

The mild stressor model described in the present study differed from other mild stressor models in that it produced normal adrenal glands and normal basal plasma corticosterone and ACTH levels. This model also displayed less

locomotor activity than non-stressed rats in the open field and a slightly blunted plasma ACTH response following acute restraint. It is possible that this could be a viable prenatal stress model to study subtle changes in HPA axis activity and its effects on different areas of the brain including the limbic system and basal ganglia.

However it is important to note that other factors including handling and the environment may influence the development of the HPA axis and thus its response to stressors in adult offspring.

CHAPTER 6

The effects of exercise on a mild prenatal stress model

6.1 INTRODUCTION

The exposure of the foetal brain to elevated circulating corticosterone levels during critical periods in development and maturation has effects that include a 50 % decrease in cell proliferation *(Van den Hove et al 2006).* The decrease in cell proliferation in the rat brain may result in permanent behavioural, metabolic and motor changes *(Weinstock 1997, Ward et al 2000).* The development and maturation of the neuronal circuits in the brain depends on several major factors that include genetic directives and complexitiy and degree of environmental stimulation *(Kehoe et al 2001).* Stress during gestation does not only affect the foetus but has been shown to induce lasting effects on the emotional reactivity of the dam *(Darnaudéry et al 2004),* thus necessitating cross-fostering the pups to non-stressed dams during the lactation period. Prenatal stress has been shown to result in a decrease in BDNF in adult offspring *(Van den Hove et al 2006).* Studies that investigated the pathophysiology of Parkinson's disease have mainly focused on using large doses of 6-OHDA that create large lesions in the nigrostriatal pathway that are comparable to end stage Parkinson's disease *(Henderson et al 2003, Emborg 2004),* In order to mimic preclinical Parkinson's disease, doses of 6-OHDA that create a partial lesion but can exhibit the subtle behavioural deficits associated with early disease are necessary *(Truong et al 2006).*

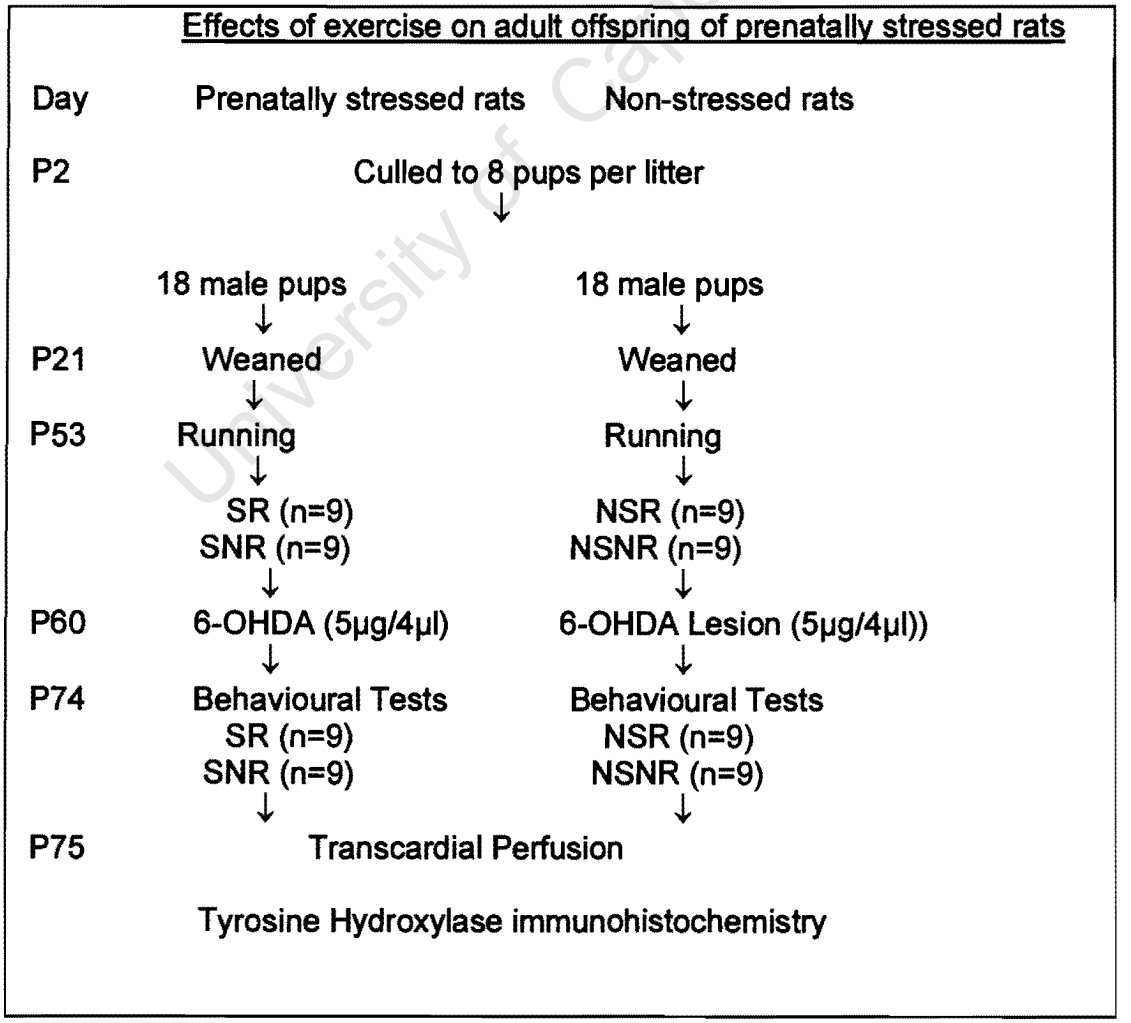
Therefore in creating a mild prenatal stress model, our aim was to look at whether exercise can reverse the vulnerability of the brain to the toxic effects of 6-OHDA by attenuating behavioural deficits in adult offspring.

6.2 MATERIALS AND METHODS

6.2.1 Mild prenatal stress model

For the mild prenatal stress model, we followed the same procedure outlined in Section 5.2.1. The rats were weaned on P21 and then housed 4 per cage until P47 in 12 hr (7am to 7pm) light-dark cycle *(Table 6.2)*. Rats were allowed free access to commercial pellet food and tap water.

Table 6.2 Flow diagram of experimental protocol showing prenatally stressed (SR) rats and non-stressed (NSR) rats that had access to running wheels and prenatally stressed (SNR) rats and non-stressed (NSNR) rats without running wheels.



6.2.2 Running experiments

On postnatal day 47, the rats housed 4 per cage were moved to a room with a 23h00-11h00 light/dark cycle. On postnatal day 53, Eighteen prenatally stressed rats and eighteen non-stressed rats were weighed and divided into two groups each. Nine prenatally stressed rats and nine non-stressed rats were placed individually into cages that had running wheels attached. The remaining rats, nine in each group were placed individually into plexiglass cages. The rats received food and water *ad libitum*. The running wheels were fitted with counters which measured the revolutions made by the rats. One complete revolution is one meter in distance. Running in the wheels was recorded daily between 10h00 and 11h00 which was 1 h before the dark cycle began. On postnatal 60, the rats in the four groups were weighed and taken to the lab where they were to undergo stereotaxic surgery.

6.2.3 Stereotaxic surgery

As 6-OHDA is also toxic to norepinephrine neurons, a norepinephrine reuptake blocker desipramine (15mg/kg, Sigma St. Louis, MO, U.S.A) was injected intraperitonially 30 min before 6-OHDA infusion. The rats were anaesthetised using a mixture of oxygen and halothane administered via a calibrated Blease Vaporiser (DATUM). After exposing the skull by making a midline incision with a scalpel, a burr-hole was constructed above the target area (see coordinates below). Both maternally separated and non-stressed rats received 6-OHDA HCl (5 µg/4 µl saline; Sigma, St. Louis, MO, U.S.A) infusion unilaterally (0.5 µl/min) using a 32G dental needle into the left MFB (4.7 mm anterior to lambda, 1.6 mm lateral to midline and 8.4 mm ventral to dura, *Paxinos et al 1986*, coordinates, *Guan et al 2000*). The infusion needle was left in the medial forebrain bundle for 1 min before infusion began. After the infusion, the needle was left in the MFB for a further 5 min so as to allow time for the neurotoxin to diffuse into the tissue. The needle was then retracted and the burr-hole closed with bone wax. After suturing the wound, the rats were allowed to recover in plexiglass cages (one per cage) for two

hours in the surgical laboratory before they were returned to their respective cages. The number of revolutions produced by the rats in the cages with attached running wheels was recorded daily for a further two weeks following surgery.

6.2.4 Behavioural tests

On postnatal day 74, the rats were weighed and taken to a behavioural testing room. The rats were placed in the testing room at least one hour before testing so as to acclimatize to the new environment. Tests to be conducted included the forelimb akinesia test (step test), the limb use asymmetry test (cylinder test) and the open field test. The light in the behavioural testing room had an intensity of 48 lux. The equipment used in the tests was cleaned with alcohol between tests.

6.2.5 Step test

The step test was designed to look at movement initiation and thus measure the severity of the lesion of each limb *(Schallert et al 2000).* The rat is held by its torso such that the hind limbs are in mid air and the weight of the rat is centered over one forelimb *(Tillerson et al 2001).* To minimize head turning, the head and the forelimb not being tested are gently oriented forward by using the thumb and index finger *(Schallert et al 2005).* The length of the step taken by each forelimb was measured and recorded. Each forelimb was tested three times and the mean was recorded as the step taken by each limb.

6.2.6 Cylinder test

Each rat was placed in a plexiglass cylinder that is 30cm high by 20 cm in diameter with the bottom and top end open. A camera was placed above the cylinder to record the number of times the rat's forelimbs touched the cylinder wall and moved across the cylinder wall while still standing on its hindlimbs. The forelimb the rat preferred to use when landing on the floor was also

recorded. Each test lasted 5 minutes so as to make sure that the rats did not habituate to the cylinder and become inactive. Limb use asymmetry was scored as the percentage of left, right or both limb wall placement (touch), wall movement and floor landing *(Tillerson et al 2001, Schallert et al 2005)*. To measure the percentage preference of the rats to use the unimpaired limb we used the formula:

[(ipsi+½both) divided by (ipsi+contra+both)] X 100.

Ipsi stands for the limb ipsilateral to the lesioned hemisphere which is the unimpaired limb and contra (contralateral limb) is the limb contralateral to the lesioned hemisphere and therefore the impaired limb. After the test, the rats were returned to their cages in the holding room where they remained for 2 h before being tested in the open field apparatus.

6.2.7 Open field apparatus

The open field apparatus is used to measure activity of the rats in a novel environment *(Colorado et al 2006)* which includes locomotor activity (line crossing), exploration (rearing) and fear and anxiety (centre square entries) *(McFadyen-Leussis et al 2004)*. The open field activity box measured 1 m X1 m X 0.5 m with charcoal grey fiberglass flooring and the four lateral sides painted cream. The inner zone measured 0.7 m X 0.7 m. Each rat was tested for a period of 5 min. To aid with the analysis, a video camera was used to record behavioral activity. Following the tests, the rats were returned to the animal facility and then on postnatal day 75 were sacrificed by transcardial perfusion and the brains stored for tyrosine hydroxylase immunohistochemistry.

6.2.8 Transcardial Perfusion and tyrosine hydroxylase immunohistochemistry

The technique used in transcardial perfusion and tyrosine hydroxylase immunohistochemistry was described in Sections 2.2.4-2.2.8.

6.3 STATISTICAL ANALYSIS

Graph Pad Prism 4 was used for statistical analysis. ANOVA was used to analyse the data and when significant differences were found ($p<0.05$), post hoc comparison using Tukey's Multiple Comparison Test was performed. Results are reported as mean ± standard error of the mean (SEM).

6.4 RESULTS

6.4.1 Locomotor Activity

There was no significant difference between the mean distance travelled by the prenatally-stressed and the non-stressed rats in cages with attached running wheels *(Table 6.4.1, Figure 6.4.1)*. The mean number of revolutions of the running wheels increased steadily from day 1 until day 7. Following stereotaxic surgery (day 7), there was a dramatic decrease in the mean number of revolutions travelled by the rats on day 8. The non-stressed rats and the prenatally-stressed rats 3 days later (day 10) achieved pre-lesion levels of activity in the running wheels.

Table 6.4.1 Mean daily distance run by prenatally stressed and non-stressed rats that had access to running wheels.

	Daily distance travelled (m)	
Day	Prenatally-stressed	Non-stressed
1	465 ± 95.9	330 ± 46.4
7	1496 ± 190	838 ± 125
8	232 ± 57.1	90.7 ± 29.4
10	1062 ± 257	842 ± 164
13	1673 ± 487	1454 ± 316

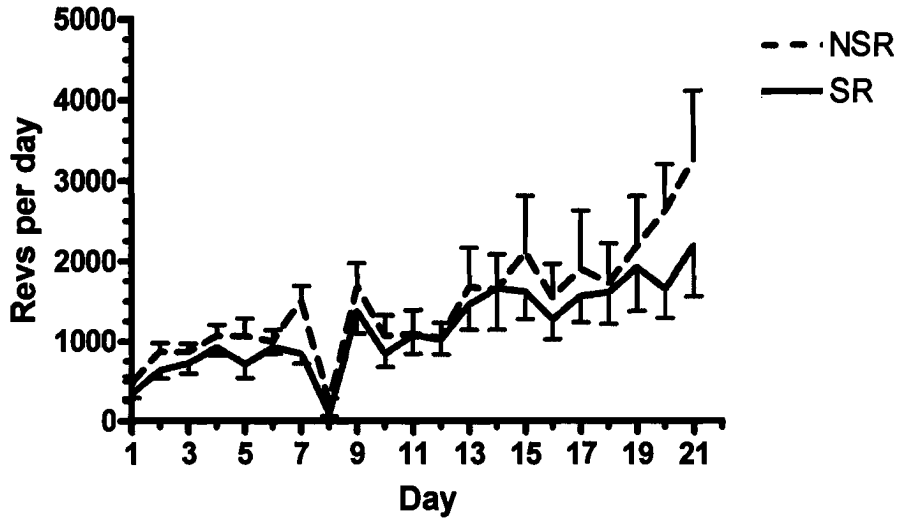


Figure 6.4.1 Mean daily distance run by prenatally-stressed (SR) rats (n=9) and non-stressed (NSR) rats (n= 9). Data reported in Table 6.4.1.

6.4.2 Rat Weights

On week 1 (PND53) the day the rats were placed individually into their respective cages, there was no significant difference between the weights of prenatal-stressed and non-stressed rats. On weeks 2, 3 and 4, there was no significant difference between the weights of the prenatally stressed (SR) rats, non-stressed (NSR) rats in cages attached to running wheels and in the weights of the prenatally stressed (SNR) rats and non-stressed (NSNR) rats without running *(Table 6.4.2 , Figure 6.4.2)*.

Table 6.4.2 Weight of the rats before lesion (week 1), day of lesion (week 2), one week after lesion (week 3) and on the day of the behavioral tests (week 4).

	Weight (g)			
Week	NSR	SR	NSNR	SNR
1:	220 ± 8.60	234 ± 9.58	205 ± 15.4	228 ± 9.86
2:	239 ± 4.99	248 ± 10.3	251 ± 10.3	266 ± 9.45
3:	294 ± 4.68	301 ± 9.80	284 ± 10.0	309 ± 10.4
4:	323 ± 4.71	326 ± 8.60	317 ± 8.00	332 ± 11.3

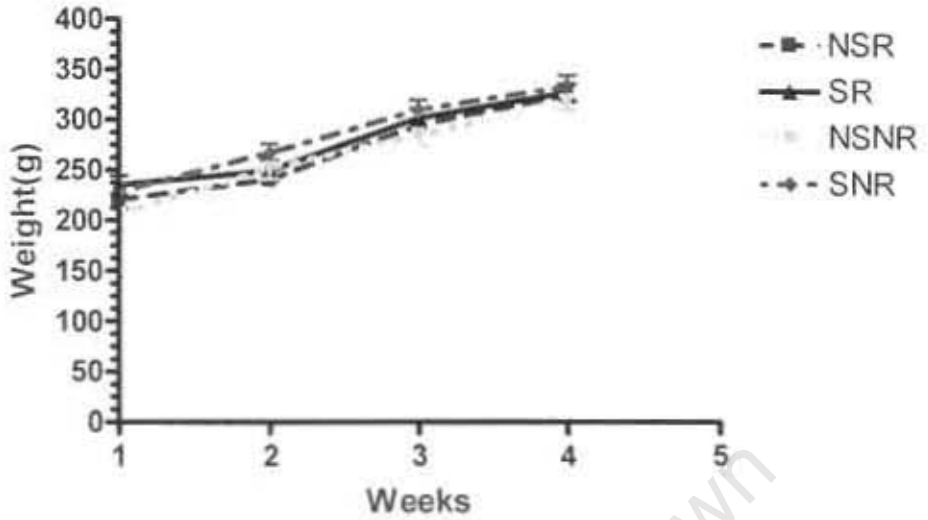


Figure 6.4.2 Weight of prenatally-stressed (SR) rats (n=9), non-stressed (NSR) rats (n=9) in running wheels and prenatally stressed (SNR) rats (n=9), non-stressed (NSNR) rats (n=9) in plexiglass cages. Data reported in Table 6.4.2.

6.4.3 Step test

The step taken by the unimpaired limb (L) whose motor functions are controlled by the non-lesioned hemisphere was significantly shorter than the step taken by the impaired limb (R) that is controlled by the lesioned hemisphere in all four groups *(Table 6.4.3 , Figure 6.4.3)*. The step taken by the impaired limb (R) of the SR rats was significantly longer than the step taken by the impaired limb of the NSR rats but was significantly shorter than the step taken by the SNR rats *(Table 6.4.3 , Figure 6.4.3)*. The step taken by the impaired limb (R) of the NSR rats was significantly shorter than the step taken by the impaired limb in NSNR rats.

Table 6.4.3 Average length of step (mm) taken by each limb. *(NSR (R) vs SR (R), p<0.001), **(NSR (R) vs NSNR (R), p<0.001) and ***(SR (R) vs SNR (R), p<0.001).

Step-length (mm)			
NSR (L)	SR (L)	NSNR (L)	SNR (L)
50 ± 1.00	50.9 ± 1.00	49.2 ± 1.43	54.6 ± 1.64
NSR (R)	*SR (R)	NSNR (R)	SNR (R)
70.6 ± 1.05	81.9 ± 1.34*	93.2 ± 1.73**	94.7 ± 1.63***

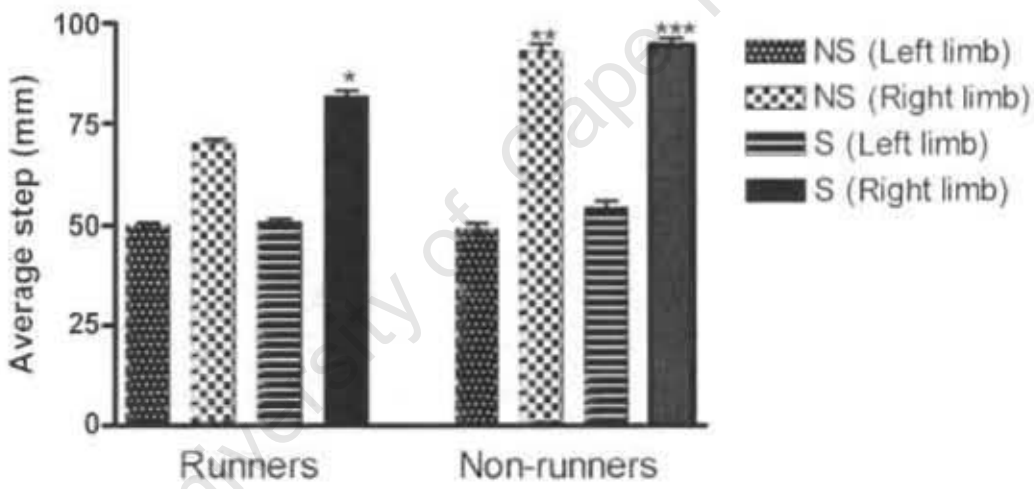


Figure 6.4.3 Average length of step taken by NSR rats (n=9), SR rats (n=9), NSNR rats (n=9) and SNR rats (n=9). L represents the left forelimb and R is the right forelimb. L vs R, p<0.001 in all groups. *(NSR (R) vs SR (R), p<0.001), **(NSR (R) vs NSNR (R), p<0.001) and ***(SR (R) vs SNR (R), p<0.001). Data reported in Table 6.4.3.

6.4.4 Cylinder Test

6.4.4.1 Wall touch

The NSR rats used the unimpaired forelimb significantly less than the NSNR *(Table 6.4.4.1, Figure 6.4.4.1)* when touching the wall of the cylinder. There was no significant difference between the preferred use of the unimpaired limb by the stressed rats *(Table 6.4.4.1, Figure 6.4.4.1)*.

Table 6.4.4.1 Percentage preference to use the unimpaired limb when touching the wall of the cylinder. *(NSR vs NSNR, $p<0.05$).

% use of the unimpaired limb when touching the wall of the cylinder			
NSR	SR	NSNR	SNR
49.8 ± 5.31	59.7 ± 4.39	68.6 ± 5.51*	76.5 ± 3.20

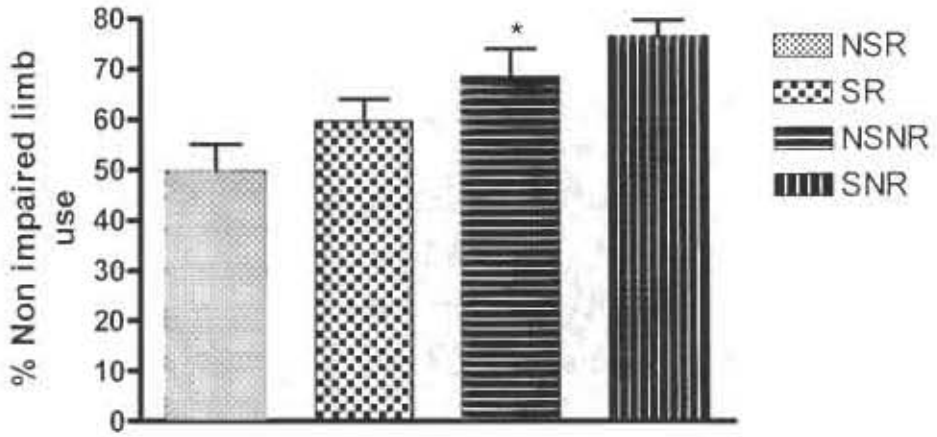


Figure 6.4.4.1 The number of times the rat preferred to use the unimpaired limb when touching the wall of the cylinder while the rat is standing on its hindlimbs expressed as a percentage of the total number of times it touched the wall of the cylinder. NSR, SR, NSNR and SNR, all n=9. *(NSR vs NSNR, $p<0.05$). Data reported in Table 6.4.4.1.

6.4.4.2 Wall movement

The non-stressed rats without running wheels (NSNR) preferred to use the unimpaired limb more than the non-stressed rats (NSR) with access to running wheels did when moving across the wall of the cylinder *(Table 6.4.4.2, Figure 6.4.4.2)*. The stressed rats without running wheels (SNR) also preferred to use the unimpaired limb more than the stressed rats with access to running wheels (SR) did when moving across the wall of the cylinder *(Table 6.4.4.2, Figure 6.4.4.2)*. There was no significant difference in limb use preference between the NSR and SR rats and between the NSNR rats and SNR rats when moving across the wall of the cylinder *(Table 6.4.4.2, Figure 6.4.4.2)*.

Table 6.4.4.2 Percentage preference to use the unimpaired limb when moving across the wall of the cylinder. *(NSR vs NSNR, p<0.05), **(SR vs SNR, p<0.01).

% use of the unimpaired limb when moving across the wall of the cylinder			
NSR	SR	NSNR	SNR
50.5 ± 6.34	61.8 ± 2.44	73.4 ± 4.92*	93.0 ± 6.90**

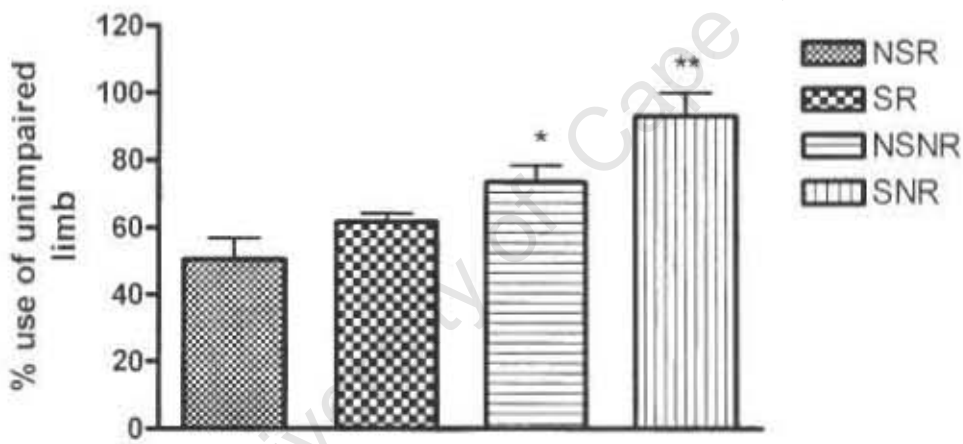


Figure 6.4.4.2 The number of times the rat preferred to use the unimpaired limb when moving across the wall of the cylinder while the rat is standing on its hindlimbs expressed as a percentage of the total number of times it moved across the wall of the cylinder NSR ,SR, NSNR and SNR, all n=9. *(NSR vs NSNR, p<0.05), **(SR vs SNR, p<0.01). Data reported in Table 6.4.4.2.

6.4.4.3 Landing

The NSNR rats preferred to use the unimpaired limb more than the NSR did when landing on the floor of the cylinder after exploring the wall of the cylinder *(Table 6.4.4.3, Figure 6.4.4.3)*.

Table 6.4.4.3 Percentage preference to use the unimpaired limb when landing on the floor of the cylinder. *(NSR vs NSNR, $p<0.05$).

% use of the unimpaired limb when landing on the floor of the cylinder			
NSR	SR	NSNR	SNR
52.1 ± 3.69	60.8 ± 4.63	69.6 ± 2.74*	73.7 ± 4.32

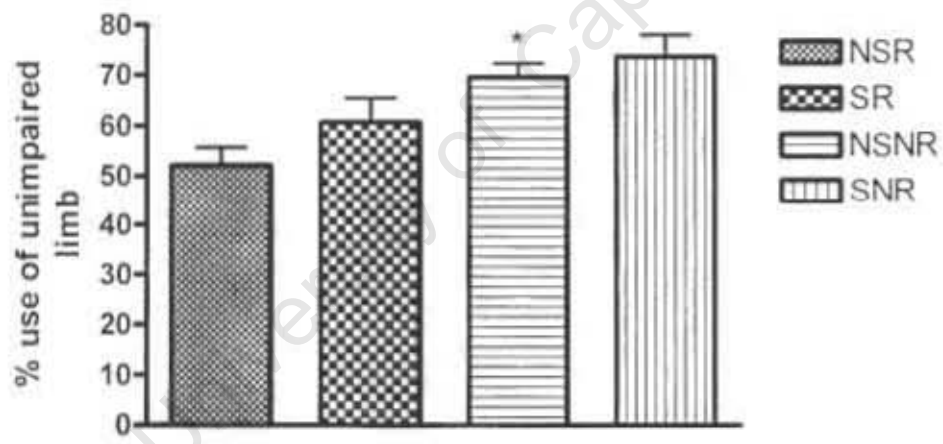


Figure 6.4.4.3 The number of times the rat preferred to use the unimpaired limb when landing on the floor of the cylinder after exploring the cylinder wall expressed as a percentage of the total number of times landed on the floor. NSR, SR, NSNR and SNR, all n=9. *(NSR vs NSNR, $p<0.05$). Data reported in Table 6.4.4.3.

6.4.5 Open field test

6.4.5.1 Total distance covered

The mean distance covered by the NSR rats was significantly greater than the distance covered by the SR rats and SNR rats *(Table 6.4.5.1, Figure 6.4.5.1)*. The mean distance covered by the NSNR rats was significantly more than the distance covered by SNR rats *(Table 6.4.5.1, Figure 6.4.5.1)*. There was no significant difference between the mean distance covered by the NSR and the distance covered by the NSNR rats *(Table 6.4.5.1, Figure 6.4.5.1)*.

Table 6.4.5.1 Mean total distance covered by the rats in the open field.
* (SR vs NSR, $p<0.05$), **(NSNR vs SNR, $p<0.05$) and ***(NSR vs SNR, $p<0.05$).

Total Distance (mm)			
NSR	SR	NSNR	SNR
4820 ± 450*	2950 ± 810	4750 ± 150**	2850 ± 200***

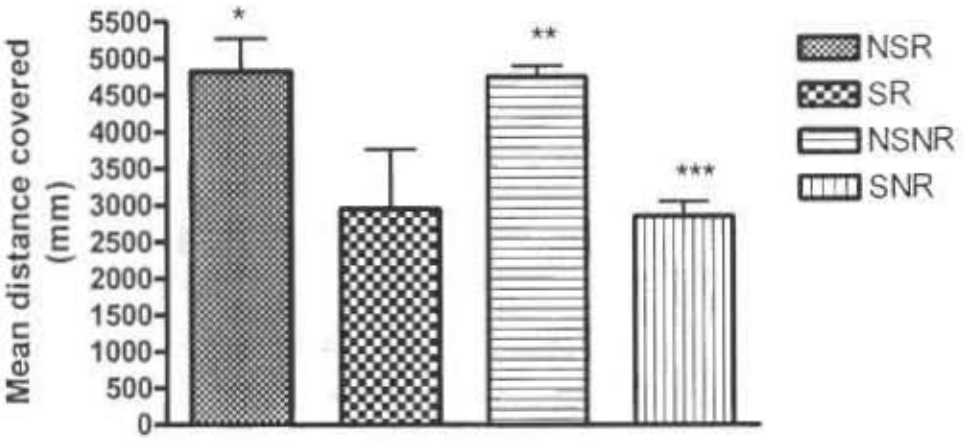


Figure 6.4.5.1 Mean total distance covered by the rats that had access to running wheels (NSR, n=9), (SR, n=9) and rats that were in plexiglass cages (NSNR, n=9), (SNR, n=9). * (SR vs NSR, $p<0.05$), **(SNR vs NSNR, $p<0.05$) and ***(NSR vs SNR, $p<0.05$). Data reported in Table 6.4.5.1.

6.4.5.2 Rearing in the open field

There was no significant difference between the number of times the NSR, SR, NSNR and SNR rats reared during the 5-min test in the open field *(Table 6.4.5.2, Figure 6.4.5.2)*.

Table 6.4.5.2 Number of times the rat reared while in the open field.

Mean number of rears			
NSR	SR	NSNR	SNR
6.11 ± 1.27	5.11 ± 1.02	4.67 ± 1.28	4.33 ± 1.00

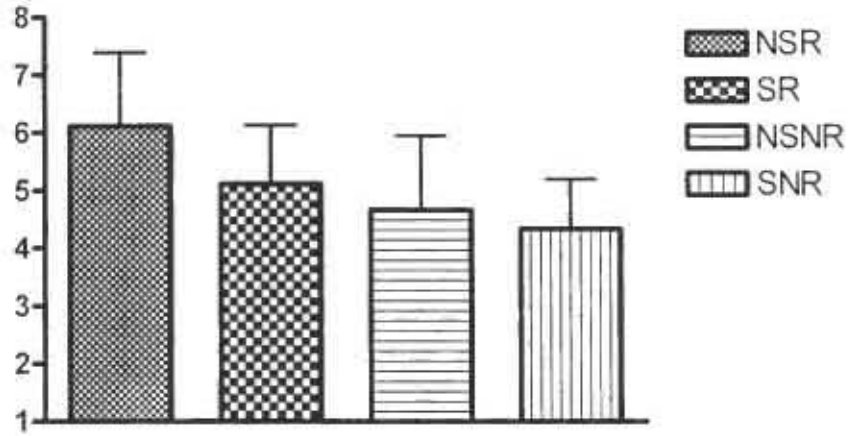


Figure 6.4.5.2 The number of rears the NSR, SR, NSNR and SNR rats (all n=9) made in a 5-min interval in the open field. Data reported in table 6.4.5.2.

6.4.5.3 Entries into the inner zone of the open field

The NSR rats entered the inner zone of the open field significantly more than the SNR rats *(Table 6.4.5.3, Figure 6.4.5.3)*. There was no significant difference between the number of times the other rats entered the inner zone of the open field during the 5-min test in the open field.

Table 6.4.5.3 The number of times the rats entered the inner zone of the open field. *(NSR vs SNR, $p<0.05$).

Entries into the inner zone of the open field			
NSR	SR	NSNR	SNR
3.89 ± 1.00	2.33 ± 1.11	2.22 ± 1.00	0.11 ± 0.11*

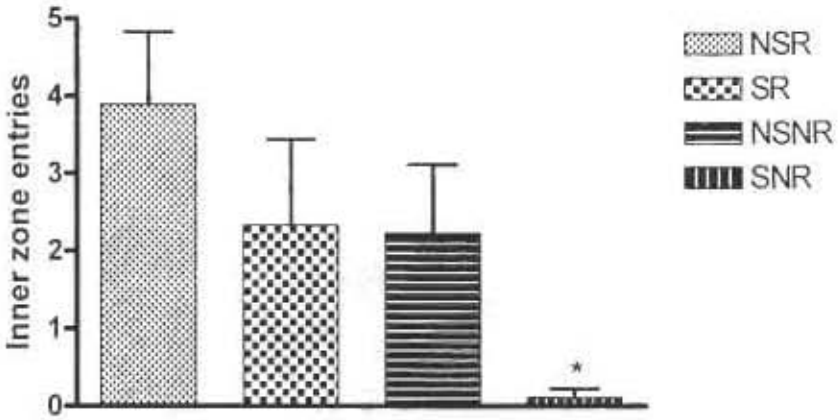


Figure 6.4.5.3 The number of times the NSR ,SR, NSNR and SNR (all n=9) rats entered the inner zone of the open field. *(NSR vs SNR, $p<0.05$). Data reported in Table 6.4.5.3.

6.4.6 Tyrosine hydroxylase immunohistochemistry

Dopamine neuron destruction in the lesioned hemisphere was calculated as a percentage of the number of tyrosine hyroxylase positive cells in the non-lesioned hemisphere. Dopamine neuron destruction in the lesioned hemisphere of NSR rats was significantly less than the dopamine neuron loss in the lesioned hemispheres of the NSNR and SNR rats *(Table 6.4.6, Figure 6.4.6)*. The dopamine neuron destruction in the lesioned hemisphere of the SR rats was significantly less than in the SNR rats but was not significantly different from the NSR rat *(Table 6.4.6, Figure 6.4.6)*.

Table 6.4.6 The percentage of dopamine neuron destruction in lesioned hemispheres of the NSR, SR, NSNR and SNR rats. *(SNR vs SR, p<0.05), **(NSR vs NSNR, p<0.05), ***(NSR vs SNR, p<0.001).

% dopamine destruction in the substantia nigra of the lesioned hemisphere.			
NSR	SR	NSNR	SNR
50.8 ± 5.11	60.77 ± 3.82*	70.53 ± 4.39**	81.07 ± 4.81***

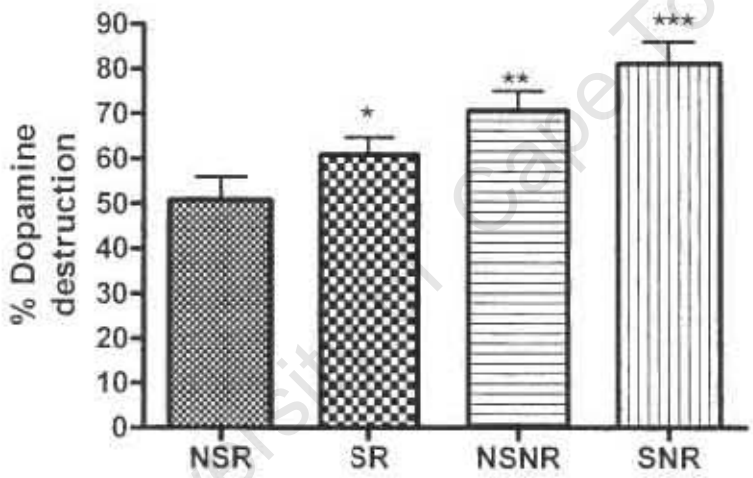


Figure 6.4.5.4 The percentage of dopamine neuron destruction in lesioned hemispheres of the NSR, SR, NSNR and SNR rats, all n=9. *(SNR vs SR, p<0.05), **(NSR vs NSNR, p<0.05), ***(NSR vs SNR, p<0.001). Data reported in Table 6.4.5.4

6.5 DISCUSSION

The results of this study suggest that exercise has a neuroprotective effect on dopamine neurons in the substantia nigra of unilateral 6-OHDA infused rats that had access to free running wheels (non-stressed runners). The beneficial effects of exercise can also be seen in the improvement in motor control in the step test and the symmetrical use of both forelimbs in the cylinder test by the non-stressed runners. In adult offspring of prenatally stressed rats, exercise improved motor control and wall exploration in the cylinder test but did not improve anxiety-like behaviour in the open field.

In the present study, the mean daily distance covered by non-stressed runners and stressed runners increased steadily from day 1 until day 7 the day of the lesion. Following the decrease in mean revolutions after unilateral 6-OHDA infusion, both the non-stressed and the stressed runners took 3 days to reach pre-lesion running distances and ran at similar mean daily revolutions until day 21 of wheel running. In the step test, the mean step taken by the non-stressed runners was significantly shorter than the mean step taken by the rats in the other groups and the mean step taken by the stressed runners was significantly shorter than the mean step taken by the stressed non-runners. The step test is used to model movement initiation involving weight shifts in Parkinson's disease and is sensitive to direct dopamine agonists in partial dopamine neuron degeneration *(Schallert et al 2000)*. The step test assesses the capacity to regain postural stability and center of gravity when rapid weight shifts are imposed *(Schallert et al 2000)*. Studies have shown that 6-OHDA lesioned animals tend to drag or brace the impaired limb rather than make catch up steps *(Schallert et al 2000, Olsson et al 1995, Lindner et al 1995)*. However the injection of direct agonists permits adequate/normal stepping *(Olsson et al 1995, Lindner et al 1995)*. As dopamine agonists were not used to achieve normal weight shifting movements in the present study, the significantly shorter step length of the non-stressed runners might suggest that dopamine degeneration in the nigrostriatal pathway of these rats was not severe. In the cylinder test which analyses forelimb use for postural support *(Schallert et al 2000, Tillerson et al 2001)* we found that the non-stressed

runners did not show a bias towards using the unimpaired limb. *Schallert et al (2000)* suggests that rats sustaining 30-70% dopamine depletion use the unimpaired limb independent of the impaired limb significantly more than rats with greater than 80% dopamine lesions especially when landing. This was the case in the present study except in the non-stressed runners which did not show a bias towards using the impaired or unimpaired limb. The preference for using the unimpaired limb is thought to be either due to the absence of recovery following injury or due to degeneration continuing at a faster rate than ongoing plasticity resulting in a decreased ability to control movement in the impaired limb *(Schallert et al 2000)*. However in the present study the dopamine neuron destruction in the substantia nigra of the non-stressed runners was 51% suggesting that according to *Schallert et al 2000*, the non-stressed runners should have used the unimpaired limb more than the injured limb when landing on the floor of the cylinder. However other studies have shown that exercise abolishes the forelimb use asymmetries associated with unilateral MFB 6-OHDA infusions *(Tillerson et al 2001, 2002)*. The presence of asymmetry in the cylinder test in stressed-runners suggests that the beneficial effects of exercise were not as prominent as in the non-stressed runners. High levels of circulating corticosterone have been shown to have an inhibitory effect on the release of neurotrophic factors *(Smith 1996, Chao et al 1994, Schaaf et 1997)*. In a previous study (Section 5.84-5), there was no significant difference between the basal corticosterone levels of the non-stressed and prenatally stressed rats. Therefore in the present study, the greater dopamine neuron destruction in the substantia nigra of the lesioned hemisphere of prenatally stressed runners might not be due to high corticosterone levels inhibiting the expression of neurotrophic factors. However in some prenatal stress models, increases in circulating corticosterone are evident after exposure to an acute stressor *(Lesage et al 2000, Chapter 5)* and therefore corticosterone levels might be increased after stereotaxic surgery. Other studies have shown that neurotrophic factors such as BDNF are decreased in offspring of rats that were prenatally stressed *(Van den Hove et al 2006)*. The increased circulating corticosterone levels in the presence of decreased BDNF levels might result in greater destruction of dopamine neurons in the substantia nigra. Exercise has been shown to

increase GDNF levels in exercising rats following 6-OHDA lesion *(Cohen et al 2003)*. In the present study, stressed runners had significantly more dopamine neuron destruction in the substantia nigra than non-stressed runners but dopamine destruction in the stressed runners was not different from that seen in the non-stressed non-runners. This might suggest that exercise is able to increase the neuroprotective factors to normal levels in stressed runners. Therefore exercise might provide neuroprotection in stressed runners but at lower levels than in non-stressed runners or that the stressed rats are more susceptible to brain injury.

When assessing the number of tyrosine hydroxylase positive cells in the substantia nigra of the lesioned hemispheres, the non-stressed runners, stressed runners and non-stressed non-runners fall within the range of non-severely (41-79%) lesioned rats whereas the stressed non-runners fell in the severely lesioned rat catergory (80-99%), *(Schallert et al 2000)*. In assessing anxiety-like behaviour in the open field *(McFadyen-Leussis et al 2004)*, the non-stressed rats in both groups were more active in the open field than the stressed non-runners and the stressed runners were more active than the stressed non-runners. However there was no significant difference between the locomotor activity of the stressed runners and non-stressed non-runners in the open field. In the mild stress model proposed in CHAPTER 5, mildly-stressed rats showed anxiety-like behaviour in the open field therefore the absence of locomotor activity differences between stressed runners and non-stressed non-runners might suggest that exercise cancelled the anxiety-like behaviour associated with open field exploration. It must be noted that most of the running done by the rats in the open field was along the wall of the open field and there was no difference in the number of times the rats ventured into the more open inner zone of the open field except between the non-stressed runners and the stressed non-runners. Therefore the absence of anxiety-like behaviour in the stressed runners might be due to adapting to the exercise regimen than to the inhibition of the anxiety-like behaviour associated with prenatal stress. The severity of the lesion in the stressed non-runners (>80%) could mean that the rats were less inclined to explore the open field due to locomotor activity deficits rather than anxiety-like behaviour. It must be noted

that the rats were tested in their dark cycle as Sprague Dawley rats tend to be in active during the light cycle *(Schallert et al 2006)*.

6.6 CONCLUSION

By using small doses of 6-OHDA, we were able to create dopamine neuron destruction more representative of early Parkinson's disease *(Truong et al 2006)*. This made it possible to unmask the beneficial effects of exercise in non-stressed rats. In a prenatal stress rat model, injecting a small dose of 6-OHDA resulted in a lesion more consistent with larger doses of 6-OHDA *(Truong et al 2006)* with dopamine neuron destruction equivalent to the destruction seen when a higher dose of 6-OHDA was used (Chapter 2), thus implying that the prenatally stressed rats are more vulnerable to the toxic effects of 6-OHDA than non-stressed rats however with voluntary exercise, substantia nigra dopamine destruction and the asymmetrical behaviour associated with a Parkinsonian rat model can be reversed or cancelled. Therefore trauma to the substantia nigra might increase the susceptibility to developing Parkinson's disease in people or animals that were exposed to prenatal stress in utero.

CHAPTER 7

Effects of Exercise on the HPA Axis of Juvenile Rats that were Maternally Separated

7.1 INTRODUCTION

Mild prenatal stress has been shown to have detrimental long term effects on brain neuron survival *(Chapter 6).* Both prenatal and postnatal stress have been shown to have long-lasting effects on the brain *(Sections 1.7, 1.8).* Therefore this raises the question of whether mild stress experienced in the early postnatal period could produce similar deficits.

Repeated short-term separation of lactating pups from their dams in the first two weeks after birth has been used as a model for maternal separation *(Section 1.83).* The effect of separation during this period is long-lasting and results in neuroendocrine abnormalities in adult offspring *(Daniels et al 2003, Meaney et al 1989, Kalinichev et al 2002).* These neuroendocrine abnormalities are thought to be due to a high expression of CRF mRNA that results in higher plasma corticosterone levels following exposure to an acute stressor *(Plotsky et al 1993).* In a previous study *(Chapter 4),* we have shown that normal Sprague Dawley rats that had access to running wheels did not show an increased corticosterone response to an acute stress. Studies of HPA axis activation in maternally separated rats have focused mainly on pups in the stress-hyporesponsive period (P 4-14) or soon after (P18-21) and in adult rats (>P 60) (SECTION 1.4.1) but have not looked at the effects of voluntary exercise on the regulation of the HPA axis.

Therefore in the present study we aimed to investigate whether the HPA axis response to an acute stress in maternally separated 7 week-old rats differed from that observed in non-stressed rats and whether exercise is able to cancel the altered stress response associated with maternal separation.

7.2 MATERIALS AND METHODS

7.2.1 Breeding

Twenty female Sprague Dawley rats were placed individually into plexiglass cages. A male rat was placed into each cage to breed with the female and was removed one week later. Gestation was twenty one days long.

7.2.2 Maternal separation stress

On postnatal day 1 (P1) pups were culled to 8 male pups per litter. As lactation provides optimal nutrition and pup-dam bonding *(Lau et al 2004),* in litters that had less than 8 male pups, female pups were added to make up the difference *(Table 7.2).* This was to ensure that the same number of pups was suckling from each dam so as to ensure that they received the same amount of nurturing. On P2 in half of the home cages (dam plus pups), the dam was removed from her litter and placed in a separate clean mouse cage with clean bedding *(Meaney et al 1993).* The litters were taken in their home cage to a different room where they were kept for three hours with the room temperature maintained constant between 31and 33 °C before being returned to the nursing room with the dams. The separation occurred daily between 09h00 and 12h00 until P14. The daily light/dark cycle of the animal facility (nursing room) was 07h00 to 19h00. The home cage was only cleaned once every fourth day and care was taken not to handle the pups as studies have shown that handled pups have increased glucocorticoid receptor expression in the hippocampus *(Meaney et al 2000, Smythe et al 1994).*This results in the handled pups having a reduced stress response to an acute stressor and stress hormones returning to basal levels more quickly than in non-handled rats *(Smith et al 2004).* Thus handling might cancel the effects of maternal separation in adult rats. Only half the bedding was changed during a cleaning session so as to ensure that the dam was still familiar with the odour of the home cage when being reintroduced to the litter following the three hour separation. This is done so that the dam recognizes the home cage as her

own and thus does not reject the pups. The remaining pups were kept with their dams under normal animal house conditions of 07h00-19h00 light/dark cycle and a temperature range of 21-24 ºC until weaning on PND 21. All rats were weaned on PND21 and then moved to a room with a 23h00-11h00 light/dark cycle.

Table 7.2 Flow diagram of experimental protocol. Maternally separated (MSR) rats and non-stressed rats (NSR) with access to running wheels and maternally separated (MSNR) rats and non-stressed (NSNR) rats in plexiglass cages.

Effects of exercise on the HPA axis of juvenile rats

Day		
P1	Culled to 8 pups per litter	
	↓	
	Maternally separated rats	Non-stressed rats
P2-14	25 male pups	26 male pups
	↓	↓
P21	Weaning	Weaning
	↓	↓
P28	Running wheels	Runningwheels
	↓	↓
	MSR (n=6)	NSR (n=7)
	MSNR (n=13)	NSNR (n=13)
	↓	↓
P49	ACTH & CORT assay	ACTH & CORT assay
	↓	↓
	Basal	Basal
	MSR (n=6)	NSR (n=7)
	*MSNR (n=5)	NSNR (n=7)
	+	+
	Post stress	Post stress
	MSR (n=6)	NSR (n=6)
	MSNR (n=6)	NSNR (n=6)

* Unable to collect blood from two rats.

3.3.3 Running experiments.

On postnatal day 28, the rats were weighed and placed in individual cages. Each group (maternally separated and non-stressed) was divided into two subgroups i.e those that had access to free running wheels and those that were placed in plexiglass cages with no wheels attached. Of the maternally separated rats, there were 12 rats in the group that had access to running wheels (MSR) and 13 rats in the group with no access to a running wheel (MSNR). Of the non-stressed rats, 13 rats had access to a running wheel (NSR) and 13 rats did not have access to a running wheel (NSNR). The rats received food and water *ad libitum*. The running wheels were fitted with counters which measured the revolutions made by the rats. One complete revolution is one meter in distance. Running in the wheels was recorded daily between 10h00 and 11h00 which was 1 h before the dark cycle began. On postnatal 49, the rats in the four groups were weighed and taken to the lab where they were to undergo an acute restraint stress. The rats were taken to the behavioural test laboratory at least an hour before basal blood levels were collected so as to acclimatize the rats to the new environment.

7.2.3 Stress response

On PND 49 rats from each group were decapitated and trunk blood was collected for basal corticosterone and ACTH determination. There was not enough blood for radioimmunoassay analysis on two of the rats in the MSNR group so we ended up with MSR, n=6, NSR, n=7, MSNR, n=5 and NSNR, n=7 rats in each group. The remaining rats were placed in rodent holders for a 10-min period. Trunk blood was collected 15 min post restraint (n = 6 from each group).

7.2.4 Radioimmunoassays

Plasma corticosterone levels were measured using an ImmuChem double antibody ^{125}I Corticosterone RIA kit (MP Biomedicals, LLC, Orangeburg,NY). A two-site solid phase immunoradiometric assay (IRMA) kit, EURIA-ACTH c.t. (EURO-DIAGNOSTICA,Malmö, Sweden), was used to determine the amount of ACTH in the plasma of the rats. A standard curve was generated by using standards provided in the kits. The levels of corticosterone and ACTH were calculated by interpolation of unknown values against the standard curve generated as described in Sections 3.2.5.1, 3.2.5.2.

7.3 STATISTICAL ANALYSIS.

Graph Pad Prism 4 was used for statistical analysis. ANOVA was used to analyse the data and when significant differences were found ($p<0.05$), post hoc comparison using Tukey's Multiple Comparison Test was performed. Results are reported as mean ± standard error of the mean (SEM).

7.4 RESULTS

7.4.1 Locomotor activity

There was no significant difference between the daily distances run by the rats that were maternally separated (MSR) and had access to running wheels and rats (NSR) that were in plexiglass cages *(Table 7.4.1, Figure 7.4.1)*.

Table 7.4.1 Mean daily distance run by 7 week-old maternally separated (MSR) and non-stressed (NSR) rats that had access to running wheels.

	Mean daily distance traveled (m)	
Day	Maternally separated	Non-stressed
1	597 ± 123	264 ± 44.8
7	1148 ± 193	1550 ± 406
13	1688 ± 264	2121 ± 667
21	2795 ± 577	3584 ± 1138

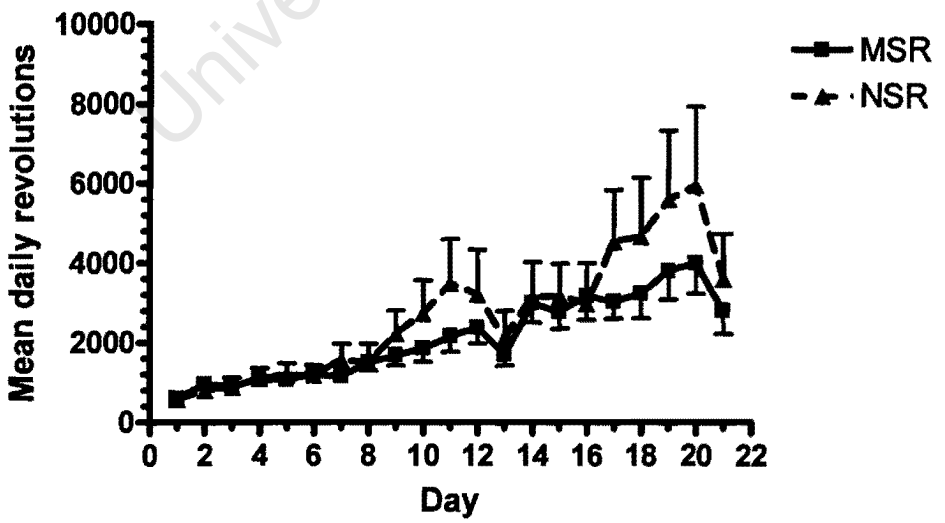


Figure 7.4.1 The mean distance travelled by 7 week-old maternally separated rats (MSR, n=12) and non-stressed rats (NSR, n=13). Data reported in Table 7.4.1.

7.4.2 ACTH analysis.

The basal ACTH concentrations of the MSR, NSR and NSNR rats were significantly lower than the basal ACTH concentration of MSNR rats. The basal levels of the of the MSR, NSR and NSNR rats were significantly lower than the respective 15 min concentrations of ACTH post restraint *(Table 7.4.2, Figure 7.4.2)*. There was no significant difference between basal and post restraint stress levels of ACTH in the MSNR rats *(Table 7.4.2, Figure 7.4.2)*.

Table 7.4.2 Plasma ACTH concentration in 7 week-old maternally separated and non-stressed rats before (basal) and after restraint stress (15 min). [1] (MSNR basal vs MSR basal, $p<0.01$), [11](MSNR basal vs NSR, $p<0.01$) and [111](MSNR basal vs NSNR, $p<0.01$). *(MSR basal vs MSR 15 min, $p<0.01$), **(NSR basal vs NSR 15 min, $p<0.05$) and ***(NSNR basal vs NSNR 15 min).

Plasma ACTH concentration (pg/ml)			
MSR (basal)	NSR (basal)	MSNR (basal)	NSNR (basal)
12.2 ± 2.18[1]	9.43 ± 1.00[11]	20.6 ± 2.01	12.6 ± 1.00[111]
*MSR (15 min)	**NSR (15 min)	MSNR (15 minl)	***NSNR (15 min)
29.3 ± 5.59	22.5 ± 2.84	19.5 ± 1.38	25.7 ± 2.80

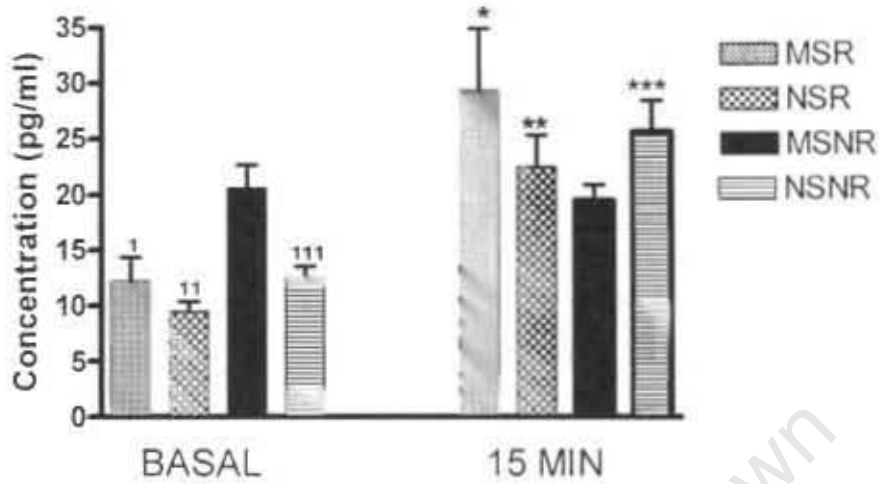


Figure 7.4.2 Plasma ACTH concentration in 7 week-old maternally separated (MSR) and non-stressed (NSR) rats housed in cages with attached running wheels before being subjected to restraint stress (basal) and 15 min post restraint stress. Maternally separated (MSNR) rats and non-stressed (NSNR) rats in plexiglass cages before being subjected to restraint stress (basal) and 15 min post restraint stress. MSR basal, n=6, NSR basal, n=7, MSNR basal, n=5, NSNR basal, n=7 and all post restraint groups, n=6. ^{1} (MSNR basal vs MSR basal, $p<0.01$), ^{11}(MSNR basal vs NSR, $p<0.01$) and ^{111}(MSNR basal vs NSNR, $p<0.01$). *(MSR basal vs MSR 15 min, $p<0.01$), **(NSR basal vs NSR 15 min, $p<0.05$) and ***(NSNR basal vs NSNR 15 min). Data reported in Table 7.4.2.

7.4.3 Corticosterone analysis

The basal corticosterone levels of the NSR and NSNR rats were significantly lower than the corticosterone levels following acute stress of NSR and NSNR rats *(Table 7.4.3, Figure 7.4.3)*. There was no significant difference between the basal and 15 min post restraint corticosterone levels in the other groups.

Table 7.4.3 Plasma corticosterone concentration in 7 week-old maternally separated and non-stressed rats before (basal) and after restraint stress (15 min). * (NSR basal vs NSR 15 min) **(NSNR basal vs NSNR 15 min).

Plasma corticosterone concentration (pg/ml)			
MSR (basal) 186 ± 31.4	NSR (basal) 150 ± 34.8	MSNR (basal) 216 ± 61.1	NSNR (basal) 187 ± 43.2
MSR (15 min) 234 ± 44.5	NSR (15 min) 306 ± 34.1*	MSNR (15 minl) 308 ± 40.8	NSNR (15 min)** 230 ± 56.2

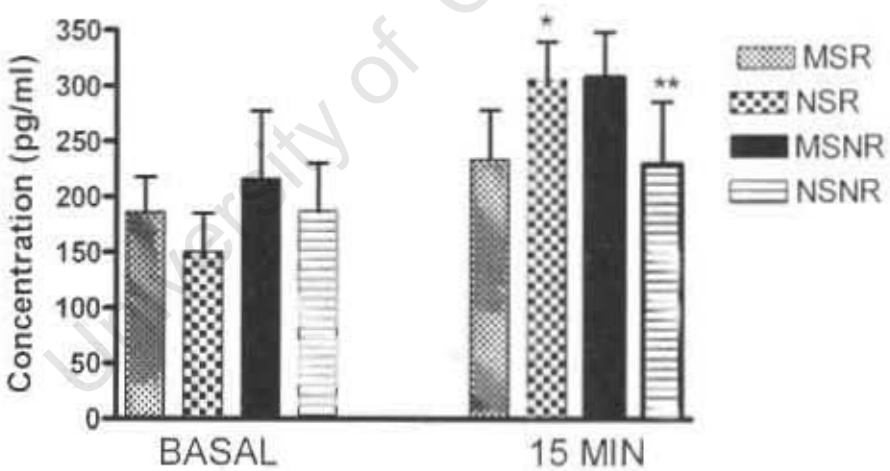


Figure 7.4.3 Plasma corticosterone concentration before (basal) and after restraint stress (15 min). For basal corticosterone levels in MSR rats (n=6), NSR rats, (n=7), MSNR rats, (n=5), and NSNR rats, (n=7). All post restraint stress groups, (n=6). * (NSR basal vs NSR 15 min) **(NSNR basal vs NSNR 15 min). Data reported in Table 7.4.3.

7.5 DISCUSSION

The results of this study suggest that exercise reverses the blunted ACTH response to an acute stress associated with a repeated short term maternal stress paradigm.

In the present study, the mean daily distance travelled by the rats in cages with attached running wheels (non-stressed runners and maternally separated runners) increased steadily from day 1 until day 21 when the rats were removed from the running wheels, with the rats travelling similar distances over the 3 week running period. The basal plasma ACTH concentration of the non-stressed rats (runners and non-runners) and the basal plasma ACTH concentration of the maternally separated runners were significantly lower than the basal plasma ACTH concentration of the maternally separated non-runners. There was a significant difference between the basal and post restraint stress plasma ACTH concentration in the non-stressed rats (runners and non-runners) and the maternally separated runners. However there was no significant difference between the basal and post restraint stress ACTH levels of the maternally separated non-runners suggesting that there was a blunted ACTH response to stress in the maternally separated non-runners in agreement with previous study by *Daniels et al (2004).*

ACTH release in the anterior pituitary gland is controlled by the pulsatile secretion of CRF into the portal system of the pituitary gland *(Section 1.6.1).* Studies have shown that in maternally separated rats, the CRF concentration in the medial eminence was increased while the pituitary CRF binding sites were reduced *(Anisman et al 1998).* However the reduction in CRF binding sites in the pituitary gland suggests that an acute increase in stress levels did not result in a normal HPA axis response. *Daniels et al (2004)* has suggested that the prolonged CRF release during maternal separation could result in the desensitisation of CRF receptors at the level of the anterior pituitary gland resulting in an abnormal HPA axis response to an acute stress. *Bremner et al (2003)* showed that adult humans that were exposed to childhood trauma had a blunted ACTH response to acute stress. However the abnormal HPA response to stress does not seem to be permanent or is sensitive to the

stress of exercise as maternally separated runners did not show a blunted ACTH response to restraint stress. The effect of exercise on the neuroendocrine system involves the secretion of corticosterone for energy production to sustain the exercise *(Ploughman et al 2006)*. Exercise has been associated with an increase in neurogenic peptides and neurotrophic factors *(Carro et al 2000, Neeper et al 1996, Windenfalk et al 1999)*. These neurotrophic factors can facilitate the sprouting of neuritis, stimulate axonal growth and axonal target finding for synaptic contacts *(Section1.4)*. However the maturation of these neurons to provide adequate adaptations for normal HPA function should take longer than 3 weeks which was the duration of the study. Therefore the absence of a blunted ACTH response to acute stress in maternally separated runners might be an exercise adaption that facilitates the release of corticosterone for energy production. In the present study, there was an elevated corticosterone response to stress in non-stressed runners and non-runners suggesting a normal response to restraint stress. However there was no significant difference between the basal and post restraint stress plasma corticosterone levels in the maternally separated runners and maternally separated non-runners. The decreased CRF binding sites in the anterior pituitary in the presence of increased CRF concentration in the medial eminence suggests maximal binding of CRF to these receptors therefore further increase in stress did not increase ACTH secretion resulting in the absence of a corticosterone response to restraint stress. However we did find an ACTH response to restraint stress in maternally separated rats suggesting that the absence of a corticosterone response to acute restraint stress might be due to decreased sensitivity of the adrenal glands to circulating ACTH levels. This might suggest that exercise adaptations in these rats include a protective mechanism to inhibit unnecessary energy utilisation so that enough energy stores are present when the rat is exercising.

7.8 CONCLUSION

The ACTH response to acute restraint stress in 7 week-old rats that were maternally separated and did not exercise was similar to that shown by *Daniels et al (2004)*. However exercise reversed the ACTH response to acute restraint stress in these rats. Studies using the maternal separation model to study the effects of childhood trauma have mainly focused on psychosomatic and affective disorders such as depression. Studies have shown that these disorders can have a neurodegenerative aspect to them as suggested by the decrease in the size of the affected areas such as the hippocampus. This suggests that the abnormal HPA axis has a role in causing progressive neurodegeneration in the hippocampus. This raises the question of whether maternal separation affects other brain areas and may thus lead to an increased vulnerability to neurodegenerative diseases such as Parkinson's disease. In a previous chapter (Chapter 6), we found that prenatally stressed rats that have also been shown to have an abnormal ACTH response to acute restraint stress were more vulnerable to the toxic insult of 6-OHDA which was not reversed by exercise. Therefore a mild early postnatal stress model such as the one used in the present study might be valuable in investigating whether perinatal stressors result in increased vulnerability to 6-OHDA infusion in a rat model for Parkinson's disease which can have implications in the ongoing investigations to the aetiology of Parkinson's disease.

CHAPTER 8

The Effects of exercise following lesion with 6-hydroxydopamine on adult rats that were maternally separated.

8.1 INTRODUCTION

Maternal separation as a stressor is used as a model to study long-term neurochemical and behavioural changes in adult rats *(Daniels et al 2003, McCormick et al 2002)*. These studies looked at behavioral and hormonal differences in adult rats that were maternally separated in the first two weeks post partum when compared to controls *(Daniels et al 2003, Meaney et al 1989)*. Injecting small doses of up to 4µg/4µl of the neurotoxic drug 6-OHDA into the medial forebrain bundle of rats can create a preclinical rat model for Parkinson's disease *(Truong et al 2006)*. Studies have shown that the injection of these small doses of 6-OHDA caused rats to display the initial motor deficits associated with early Parkinson's disease which were not present in rats with larger 6-OHDA lesions which resulted in complete dopamine dennervation with little or no recovery *(O'Dell et al 2007)*.

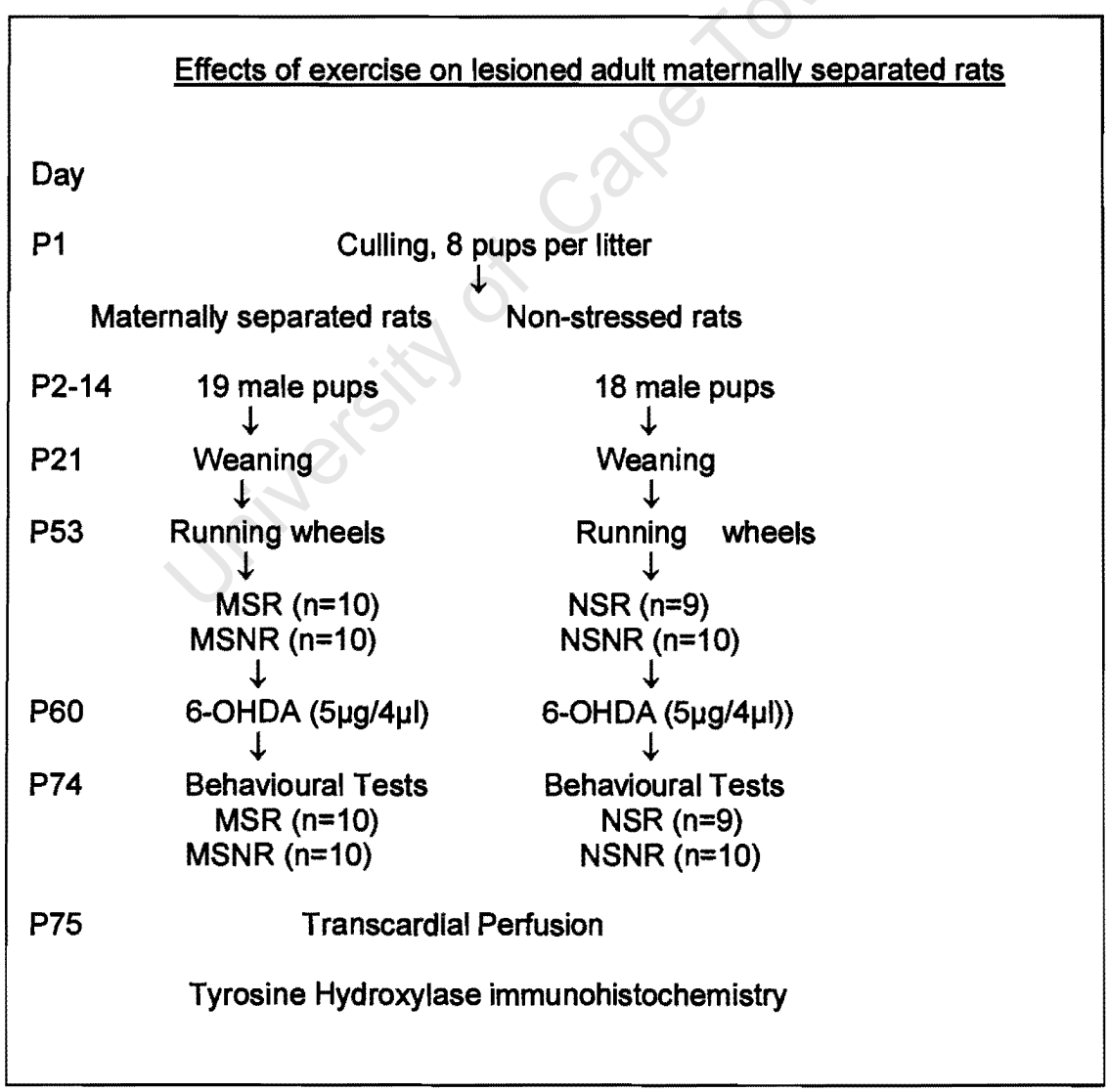
In the present study we hypothesize that early life trauma such as maternal separation leaves the striatum/substantia nigra area of the rat brain more vulnerable to the neurotoxic stress of 6-OHDA than in non-stressed rats. Therefore the aim of the study is to investigate whether:

1. Maternal separation exacerbates the size of the lesion in adult rats following injection of a small dose (5µ/4µl) into the medial forebrain bundle.
2. Voluntary exercise pre- and post-lesion can reduce this vulnerability.

8.2 MATERIALS and METHODS.

On postnatal day 1, the rats were sexed and culled to eight males per litter *(Table 8.2)*. If there were less than 8 pups per litter, the numbers were increased to eight by adding the appropriate number of females so that the same number of pups were suckling on all dams.

Table 8.2 Flow diagram of experimental protocol. Maternally separated rats with (MSR) or without (MSNR) running attached wheels and non-stressed rats with (NSR) or without (NSNR) running wheels.



8.2.1 Maternal Separation Stress

For the maternal separation paradigm, we followed the same procedure outlined in Section 7.2.1. The rats were weaned on P21 and then housed 4 per cage until P46.

8.2.2 Running experiments.

On postnatal day 46, the rats housed 4 per cage were moved to a room with a 23h00-11h00 light/dark cycle. On postnatal day 53, twenty maternally separated rats and ninenteen non-stressed rats were weighed and divided into two groups each. Ten maternally separated rats and nine non-stressed rats were placed individually into cages that had running wheels attached. The remaining rats were placed individually into plexiglass cages. The rats received food and water *ad libitum.* The running wheels were fitted with counters which measured the revolutions made by the rats. One complete revolution is one meter in distance. Running in the wheels was recorded daily between 10h00 and 11h00 which was 1 h before the dark cycle began. On postnatal 60, the rats in the four groups were weighed and taken to the lab where they were to undergo stereotaxic surgery. The rats were taken to the surgical laboratory at least an hour before surgery so as to acclimatize the rats to the new environment.

8.2.3 Stereotaxic surgery.

Stereotaxic surgery was described in SECTION 6.2.3.

8.2.4 Behavioural tests and tyrosine hydroxylase immunohistochemisrty

The behavioural tests performed included the step test, cylinder test and tests in the open field. These tests were described in Sections 6.2.5-7. The protocol for tyrosine hydroxylase immunohistochemistry was described in Sections 6.2.8-12

8.3 STATISTICAL ANALYSIS

Graph Pad Prism 4 was used for statistical analysis. ANOVA was used to analyse the data and when significant differences were found ($p<0.05$), post hoc comparison using Tukey's Multiple Comparison Test was performed. Results are reported as mean ± standard error of the mean (SEM).

8.4 RESULTS

8.4.1 Locomotor activity

There was no significant difference between the distance run by MSR and NSR rats *(Table 8.4.1, Figure 8.4.1).* The mean number of revolutions of the running wheels increased steadily from day 1 until day 7. Following stereotaxic surgery (day 7), there was a dramatic decrease in the mean number of revolutions traveled by the rats on day 8 and it took the rats 5 days to achieve pre-lesion levels of activity in the running wheels.

Table 8.4.1 Mean daily distance run by maternally separated and non-stressed rats housed in cages with attached running wheels.

	Distance traveled (m)	
Day	Maternally separated	Non-stressed
1	320 ± 46.4	264 ± 44.8
7	1880 ± 550	2350 ± 516
8	500 ± 91.3	446 ± 113
13	1765 ± 417	2170 ± 525

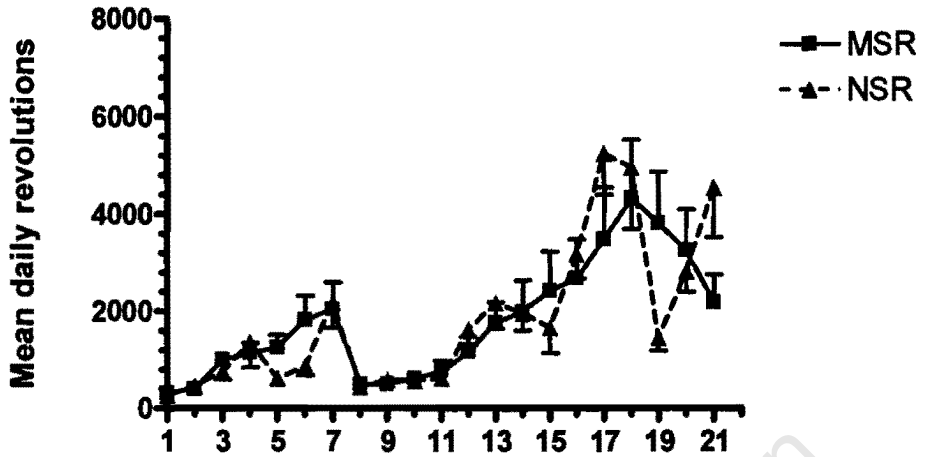


Figure 8.4.1 Mean daily distance run by maternally separated (MSR) rats (n=10) and non-stressed (NSR) rats (n= 9). Data reported in Table 8.4.1.

8.4.2 Rat Weights

On the day (P53) the rats were placed individually into their respective cages, there was no significant difference between the weights of maternally separated and non-stressed rats. At the beginning of week 2 (P60), the MSR rats weighed significantly less than the MSNR rats *(Table 8.4.2, Figure 8.4.2)*. There was no significant difference between the weights of the different groups of rats in week 4 (P74) *(Table 8. 4.2, Figure 8.4.2)*.

Table 8.4.2 Weight of the rats before lesion (week 1), day of lesion (week 2) and on the day of the behavioral tests (week 4). *(MSNR vs MSR, p<0.01, week 2).

	Weight (g)			
Week	MSR	NSR	MSNR	NSNR
1	294 ± 3.78	305 ± 4.7	320 ± 2.27	304 ± 6.95
2	284 ± 11.9*	318 ± 9.72	332 ± 2.09	316 ± 7.20
4:	333 ± 7.65	336 ± 6.98	364 ± 11.43	352 ± 8.05

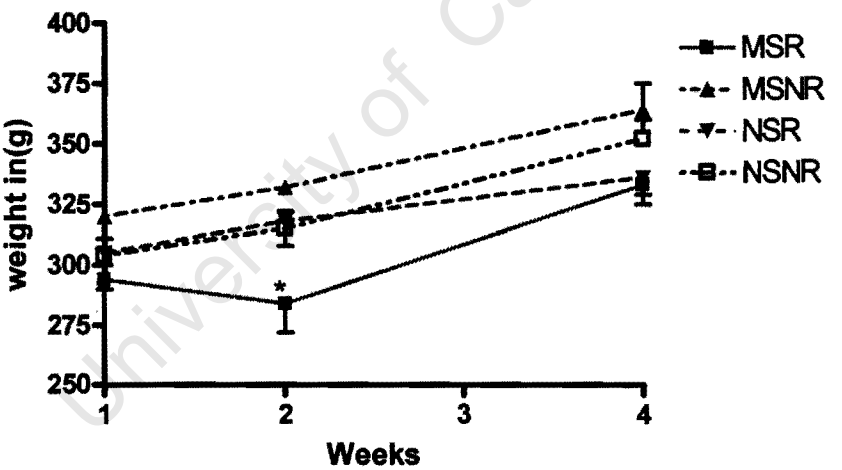


Figure 8.4.2: Weight of maternally separated rats with (MSR, n=10) or without (MSNR, n-10) running wheels and weight of the non-stressed rats with (NSR, n=9) or without (NSNR, n=10) running wheels. *(MSNR vs MSR, p<0.01, week 2). Data reported in Table 8.4.2.

8.4.3 Step test

The step taken by the unimpaired limb (L) whose motor functions are controlled by the non-lesioned hemisphere was significantly shorter than the step taken by the impaired limb (R) that is controlled by the lesioned hemisphere in all four groups *(Table 8.4.3, Figure 8.4.3)*. The step taken by the impaired limb (R) of the MSR rats was significantly longer than the step taken by the impaired limb of the NSR rats but was not significantly different from the step taken by the MSNR rats. The step taken by the impaired limb (R) of the NSR rats was significantly shorter than the step taken by the impaired limb in NSNR rats. The step taken by the impaired limb (R) of the MSNR rats was significantly longer than the step taken by the impaired limb of the NSNR rats.

Table 8.4.3 Average length of step taken by each limb. L vs R, $p<0.001$ in all groups. *(NSR (R) vs MSR (R), $p<0.001$), **(NSNR (R) vs MSNR (R), $p<0.01$), ***(NSR (R) vs NSNR (R), $p<0.001$)

Step-length (mm)			
MSR (L)	NSR (L)	MSNR (L)	NSNR (L)
50 ± 1.00	55.5 ± 0.68	53.3 ± 0.74	49.6 ± 1.03
MSR (R)	NSR(R)	MSNR (R)	NSNR(R)
82.3 ± 1.76*	67.2 ± 0.73	82.5 ± 1.17**	76.0 ± 1.43***

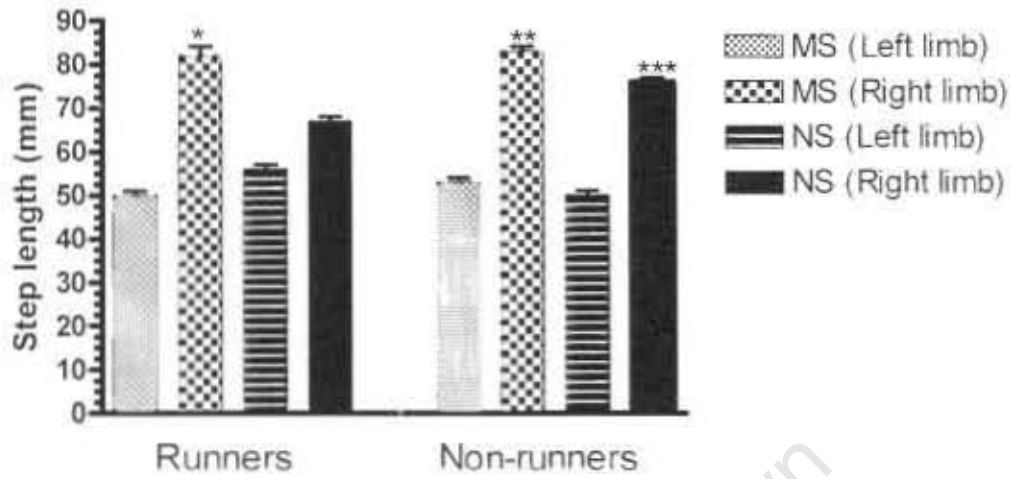


Figure 8.4.3 Average length of step taken by MSR rats (n=10), NSR rats (n=9), MSNR rats (n=10) and NSNR ras (n=10). L represents the left forelimb and R is the right forelimb. L vs R, $p<0.001$ in all groups. *(NSR (R) vs MSR (R), $p<0.001$), **(NSNR (R) vs MSNR (R), $p<0.01$), ***(NSR (R) vs NSNR (R), $p<0.001$). Data reported in Table 8.4.3.

8.4.4 Cylinder test

8.4.4.1 Wall touch

The NSR preferred to use the unimpaired limb significantly less than the MSNR rats *(Table 8.4.4.1, Figure 8.4.4.1)*.There was no significant difference between the preference of the MSR, NSR and NSNR rats to use the unimpaired forelimb when touching the wall of the cylinder *(Table 8.4.4.1, Figure 8.4.4.1)*.

Table 8.4.4.1 Percentage preference to use the unimpaired limb when touching the wall of the cylinder. *(NSR vs MSNR, p<0.05).

% use of the unimpaired limb in touching the wall of the cylinder.			
MSR	NSR	MSNR	NSNR
51.5 ± 6.34	41.8 ± 6.44	71.3 ± 6.92*	63.0 ± 6.90

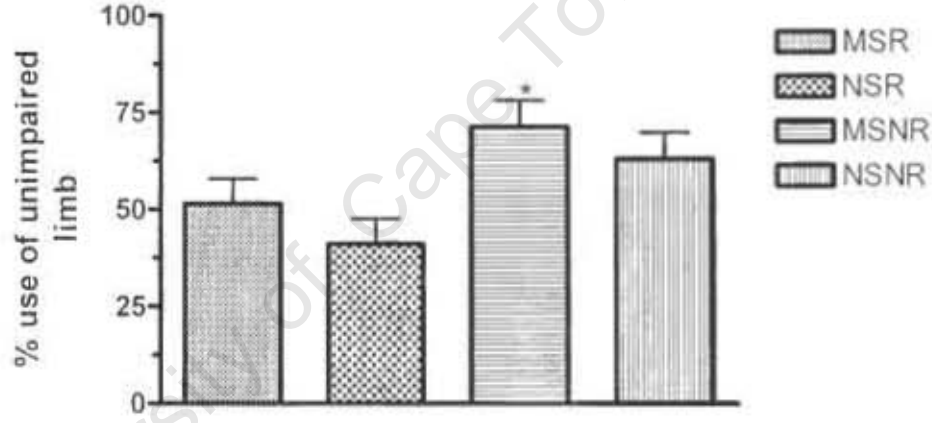


Figure 8.4.4.1 The number of times the rat preferred to use the left forelimb when touching the wall of the cylinder while the rat is standing on its hindlimbs expressed as a percentage of the total number of times it touched the wall of the cylinder (Percentage preference; Section 4.4.6). MSR rats (n=10), NSR rats (n=9), MSNR rats (n=10) and NSNR rats (n=10). *(NSR vs MSNR, p<0.05). Data reported in Table 8.4.4.1.

8.4.4.2 Wall movement

The MSR and NSR rats used the unimpaired forelimb significantly less than the MSNR and NSNR rats, respectively, when moving across the cylinder *(Table 8.4.4.2; Figure 8.4.4.2)*.

Table 8.4.4.2 Percentage preference to use the unimpaired limb when moving across the wall of the cylinder. *(MSR vs MSNR, $p<0.05$) and **(NSR vs NSNR, $p<0.05$).

% use of the unimpaired limb in moving across the wall of the cylinder			
MSR	NSR	MSNR	NSNR
53.1 ± 6.69	47.8 ± 2.63	69.6 ± 2.74*	63.7 ± 2.32**

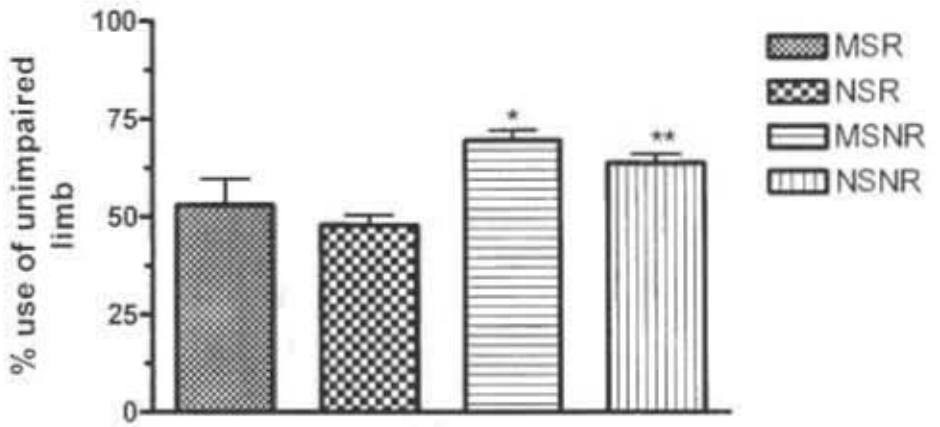


Figure 8.4.4.2 The number of times the rat preferred to use the unimpaired limb when moving across the wall of the cylinder while the rat was standing on its hindlimbs expressed as a percentage of the total number of times it used its forelimbs to move across the wall of the cylinder (Percentage preference; Section 4.2.4.6). MSR rats (n=10), NSR rats (n=9), MSNR rats (n=10) and NSNR rats (n=10). *(MSR vs MSNR, $p<0.05$) and **(NSR vs NSNR, $p<0.05$). Data reported in Table 8.4.4.2.

8.4.4.3 Landing

When landing on the floor after touching or moving across the wall of the cylinder, the MSNR rats used the unimpaired forelimb significantly more frequently than the MSR rats *(Table 8.4.4.3, Figure 8.4.4.3)*. The MSNR rats also preferred to use the unimpaired limb significantly more than the NSNR rats *(Table 8.4.4.3, Figure 8.4.4.3)*.. There was no significant difference between the number of times NSR and MSR or NSR and NSNR rats preferred to use the unimpaired limb *(Table 8.4.4.3, Figure 8.4.4.3)*.

Table 8.4.4.3 Percent preference to use the unimpaired limb when landing on the floor following cylinder wall touch and movement. *(MSNR vs NSR, p<0.01). **(MSR vs MSNR, p<0.05) and ***(MSNR vs NSNR, p<0.05)

% use of the unimpaired limb when landing on the wall of the cylinder			
MSR	NSR	MSNR	NSNR
52.6 ± 2.49*	49.8 ± 1.40	65.8 ± 5.57**	51.4 ± 1.29***

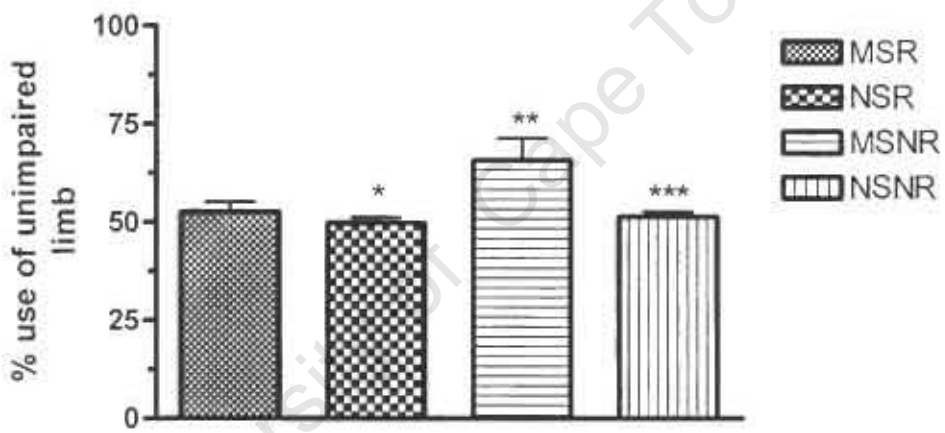


Figure 8.4.4.3 Forelimb preferred by the rat when landing on the floor expressed as a percentage of the total number of times it landed on the floor of the cylinder (Percentage preference; Section 4.4.6). MSR rats (n=10), NSR rats (n=9), MSNR rats (n=10) and NSNR rats (n=10). *(MSNR vs NSR, p<0.01). **(MSR vs MSNR, p<0.05) and ***(MSNR vs NSNR, p<0.05). Data reported in table 8.4.4.3.

8.4.5 Open field tests

8.4.5.1 Distance traveled

The mean distance covered by the NSR and NSNR rats in the open field was significantly greater than the mean distance covered by the MSNR rats *(Table 8.4.5.1, Figure 8.4.5.1)*. There was no significant difference between the mean distance covered by the NSR, NSNR and MSR rats and between the MSR and MSNR rats *(Table 8.4.5.1, Figure 8.4.5.1)*.

Table 8.4.5.1 Mean total distance covered by the rats in the open field.
* (NSR vs MSNR, p<0.001), **(NSNR vs MSNR, p<0.001)

Distance (mm)			
MSR	NSR	MSNR	NSNR
2020 ± 630	4510 ± 420	1010 ± 690*	4500 ± 405**

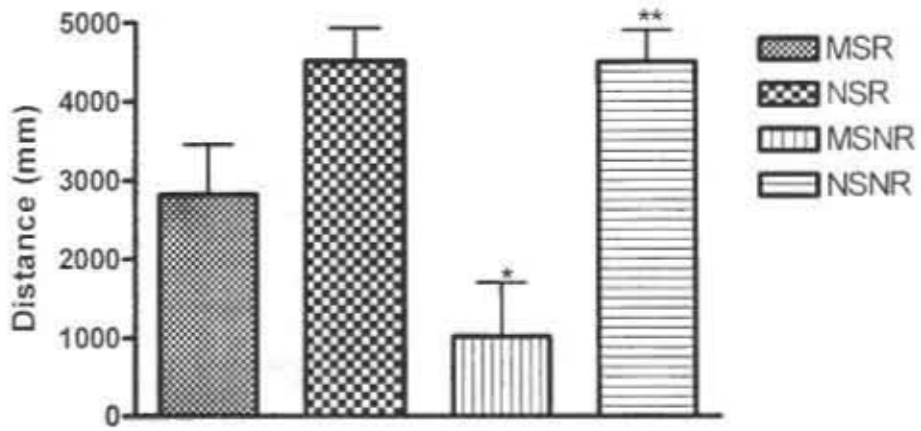


Figure 8.4.5.1 Mean total distance covered in the open field by rats that had access to running wheels (MSR, n=10), (NSR, n=9) and rats that were in plexiglass cages (MSNR, n=10), (NSNR, n=10). *(NSR vs MSNR, $p<0.001$), **(NSNR vs MSNR, $p<0.001$). Data reported in Table 8.4.5.1.

8.4.5.2 Rearing

There was no significant difference between the number of times the MSR, NSR, MSNR and NSNR rats reared during the 5-min test in the open field *(Table 8.4.5.2, Figure 8.4.5.2)*.

Table 8.4.5.2 Number of times the rat reared while in the open field.

Mean number of rears			
MSR	NSR	MSNR	NSNR
13.0 ± 2.72	10.7 ± 2.20	11.6 ± 1.33	11.0 ± 1.48

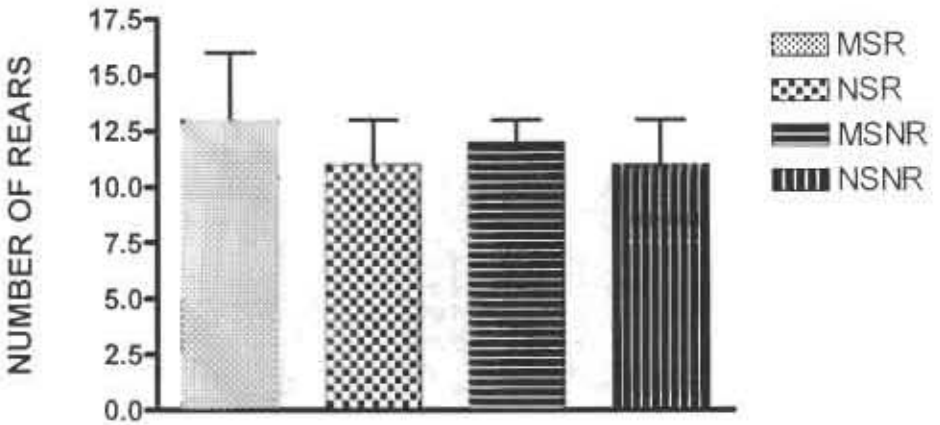


Figure 8.4.5.2 The number of rears the MSR rats (n=10), NSR rats (n=9), MSNR) rats (n=10) and NSNR rats (n=10) made in a 5-min interval in the open field. Data reported in table 8.4.5.2.

8.4.5.3 Open field test (entries into the inner zone)

There was no significant difference between the number of times the rats entered into the inner zone of the open field during the 5-min test in the open field (*Table 8.4.5.3, Figure 8.4.5.3.*

Table 8.4.5.3 The number of times the rats entered the inner zone of the open field.

Entries into the inner zone of the open field			
MSR	NSR	MSNR	NSNR
3.22 ± 0.81	3.44 ± 1.13	1.70 ± 0.72	2.60 ± 0.88

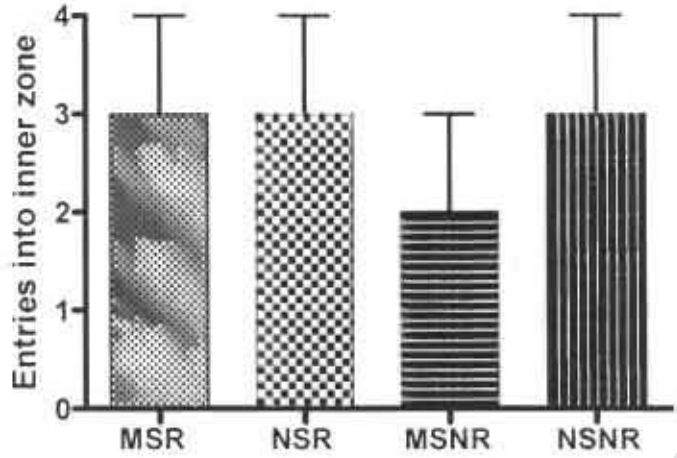


Figure 8.4.5.3 The number of entries into the inner zone of the open field by the MSR rats (n=10), NSR rats, (n=9) MSNR rats (n=10) and NSNR rats (n=10). Data reported in Table 8.4.5.3.

8.4.6. Tyrosine hydroxylase immunohistochemistry

The amount of dopamine neuron destruction (expressed as a percentage of tyrosine hydroxylase positive cells present in the lesioned hemisphere when compared to the non-lesioned hemisphere) in the substantia of the NSR rats was significantly less than the dopamine neuron destruction in the MSNR and NSNR rats *(Table 8.4.6, Figure 8.4.6)*. The amount of dopamine neuron destruction in the lesioned substantia nigra of MSR rats was significantly greater than in the MSNR rats. There was no significant difference between the number of surviving dopamine neurons in the lesioned substantia nigra of MSR and NSR rats and between the MSR and NSNR rats *(Table 8.4.6, Figure 8.4.6)*.

Table 8.4.6 The percentage of dopamine neuron destruction in the lesioned hemispheres of the MSR, NSR, MSNR and NSNR rats. *(MSNR vs NSR, p<0.01), **(MSR vs MSNR, p<0.05) and ***(NSR vs NSNR, p<0.01)

% dopamine destruction in the substantia nigra			
MSR	NSR	MSNR	NSNR
52.4 ± 5.24	46.5 ± 3.49*	69.4 ± 3.58**	68.9 ± 5.08***

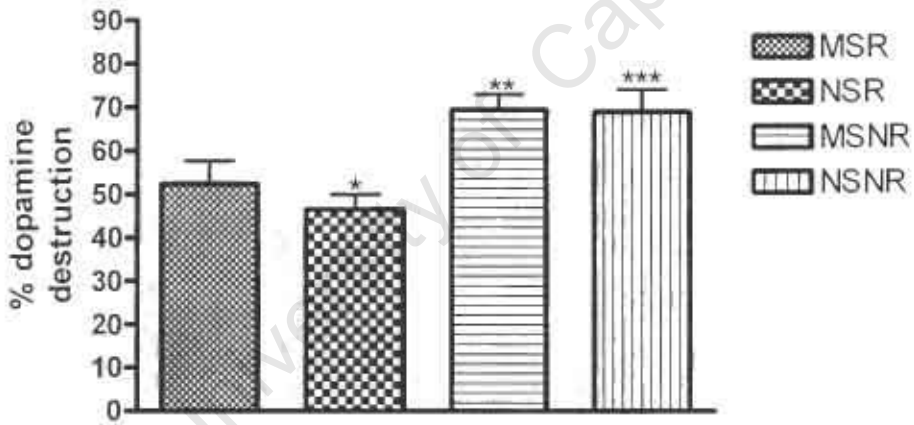


Figure 8.4.6 The percentage of dopamine neuron destruction in lesioned hemispheres of the MSR (n=10), NSR (n=9), MSNR (n=10) and NSNR (n=10) rats. *(MSNR vs NSR, p<0.01), **(MSR vs MSNR, p<0.05) and ***(NSR vs NSNR, p<0.01). Data reported in Table 8.4.6.

8.5 DISCUSSION

The results of this study suggest that in a Parkinsonian rat model adult rats that were maternally separated and were without running wheels displayed a decrease in motor control, increase in asymmetrical bias towards unimpaired forelimb use and greater dopamine neuron destruction in the substantia nigra than in non-stressed rats with running wheels. However there was no significant difference between the non-stressed rats and the maternally separated rats both with running wheels, in forelimb use asymmetry and the amount of dopamine neuron destruction in the lesioned substantia suggesting that exercise provided neuroprotection to the dopamine neurons in the substantia nigra following 6-OHDA lesion.

In the present study, rats that had access to running wheels (both non-stressed and maternally separated) increased their running activity steadily from day 1 until the day of the lesion. Following lesion, it took the rats approximately 6 days to run at pre-lesion rates after which the running activity increased steadily without any significant differences in the mean daily running activity until the end of the experiment on P74. In the second week of running, there was a significant decrease in the weight of the maternally separated rats in cages with running wheels compared to the maternally separated rats without running wheels. The weight of the rats increased gradually until there was no significant difference between maternally separated rats with and without wheels. The initial decrease in the weight of the maternally separated rats that were allowed to exercise could have been the result of be a physiological response to exercise. However the absence of a weight decrease in non-stressed rats with access to running wheels might suggest that the physiological stress of running was significantly greater in the maternally separated rats. Maternal separation stress might have altered their perception of effort and ability to cope with the stress of running. Although not significantly different, the daily running distance covered by the maternally separated rats seemed to be more than the distance covered by the non-stressed rats suggesting that more energy might have been expended by the maternally separated rats.

In the non-stressed rats with access to running wheels, there was a significant difference between the length of step taken by the impaired limb when compared to the step taken by impaired limbs of the other rats. One of the symptoms of Parkinson's disease is paucity in movement and difficulty in initiating movement *(Squires et al 2003).* The decrease in the step length taken by the impaired forelimb during the step test in non-stressed rats with running wheels might mean that the lesion size in these rats was relatively small suggesting that there were enough dopamine neurons in the nigrostriatal pathway to maintain normal control over movement. However it seems that exercise did not provide the same amount of protection in maternally separated rats as there was a significant difference between the mean step taken by the maternally separated rats and the mean step taken by the non-stressed rats. This finding cannot be explained other than to suggest that neural circuits have been altered or that this could be an anomaly in muscle strength of maternally separated rats. This could be supported by the finding that there was no significant difference between the mean step length taken by the impaired limb of the maternally separated rats that were able to exercise or not despite the presence of a significant difference in the amount of dopamine neuron destruction in the lesioned substantia nigra of these two groups of rats (to be discussed below). The presence of a significantly greater adjusting or bracing behaviour in the non-stressed and maternally separated rats without running wheels than in non-stressed runners suggests the presence of greater dopamine neuron destruction in the lesioned substantia nigra of these rats.

In the cylinder tests, the non-stressed and maternally separated rats in cages with running wheels used the unimpaired limb significantly more than the non-stressed and maternally separated rats without running wheels respectively when moving across the wall of the cylinder or when landing on the floor of the cylinder. *Tillerson et al (2001, 2002),* has shown that exercise abolishes the forelimb use asymmetries associated with unilateral 6-OHDA lesions in the

cylinder test. As the cylinder test is designed to analyze forelimb use for postural support *(Schallert et al 2000),* the absence of a significant difference between the maternally separated rats with and without running wheels in touching the wall of the cylinder might suggest that this test is not as robust as movement initiation in the wall movement test or landing. Wall movement and floor landing, require a significant weight transfer to the forelimbs whereas in the wall touch cylinder test, the hind limbs bear most of the weight as the rats can rear without touching the wall. The impaired forelimb of the maternally separated rats in cages without attached running wheel was not flaccid as shown by the ability to brace without the snout touching the table top at the end of the movement in the step test. This suggests that movements that do not require weight bearing, can be performed equally well with the impaired limb in rats with excessive dopamine neuron loss. The step test is forced movement suggesting that under normal conditions, the rat would prefer to use the unimpaired limb in excessively dopamine neuron depleted rats.

The mean distance run by the rats in the open field suggests that the maternally separated rats without running wheels exhibited more locomotor behaviour than non-stressed rats with or without running wheels. However there was no significant difference between the non-stressed and maternally separated rats with running wheels and between the maternally separated rats with and without running wheels. This might suggest that the reduction in locomotor activity in the maternally separatedrats without running wheels is due to the increased extent of dopamine neuron damage that affects motor function more than the beneficial effects of exercise. However the absence of a significant difference between the number of tyrosine hydroxylase positive cells in the lesioned substantia nigra of maternally separated and non-stressed rats without running wheels suggests that if dopamine neuron damage was the only reason for the lack of locomotor activity then the non-stressed rats without running wheels should have been similarly affected. There was no significant difference between the number of entries into the inner zone in all groups 4 groups (maternally separated and non-stressed

rats with or without running wheels) suggesting that the maternally separated rats did not exhibit anxiety-like behavior in the open field.
There was a significant difference between the extent of dopamine neuron destruction in non-stressed rats with and without running wheels and dopamine neuron destruction between non-stressed rats with running wheels and maternally separated rats without running wheels suggesting that exercise plays a role in providing neuroprotection in rats that were unilaterally lesioned with 6-OHDA into the MFB.

There was no significant difference between exercised non-stressed and maternally separated rats in the amount of dopamine destruction in the lesioned substantia nigra. Dopamine destruction in the maternally separated rats with running wheels was significantly less than the dopamine destruction in the non-stressed rats without running wheels. This suggests that exercise does provide neuroprotection in maternally separated rats. In some studies of maternal separation using the repeated three week maternal separation paradigm, corticosterone levels are significantly increased following exposure to an acute stress *(Section 1.8.3)*. This suggests that the brains of these rats might be more vulnerable to injury than normal rats as corticosterone has an inhibitory effect on the expression of neurotrophic factors that are neuroprotective or neuroregenerative in the presence of neurodegeneration *(Section 1.5)*. However studies have shown that exposure to the repeated maternal paradigm used in this study resulted in a highly significant increase in BDNF protein concentration in the hippocampus of adult rats that were maternally separated compared to non-stressed controls *(Greisen et al 2005)*. The increase in BDNF or other neurotrophic factor expression might be present in other brain regions such as the nigrostriatal pathway which would result in the presence of neuroprotection in rats that were maternally separated. The absence of a significant difference in the amount of dopamine neuron destruction as shown by the percentage of tyrosine hydroxylase positive cells remaining in the lesioned substantia nigra of the non-stressed rats and maternally separated rats without running wheels

suggests that the amount of neutrophic factor expression was similar. It is important to note that the dopamine neuron destruction in the lesioned substantia nigra of adult offspring of prenatally-stressed rats without running wheels was greater than 80% following a small dose (5µg/4µl) of 6-OHDA *(Section 6.4.6)* whereas in the present study, dopamine destruction with a similar dose of 6-OHDA was less than 70% in the non-stressed and maternally separated rats without running wheels. Interestingly the amount of dopamine neuron destruction in the present study was similar to the amount of dopamine destruction seen when a larger dose (10µg/4µl) of 6-OHDA was injected into the MFB of rats with running wheels in a previous study *(Section 2.4.3).* Due to the proximity of the MFB to the substantia nigra, injecting 6-OHDA into the MFB results in a rapid neurotoxic effect in the dopamine neuron cell bodies in the substantia nigra. If it takes 3 days for GDNF levels to reach peak values in injured substantia nigra as suggested by *Cohen et al 2003,* injecting large doses of 6-OHDA into the MFB might result in the toxic effects of this neurotoxin overcoming the neuroprotective effects of GDNF resulting in dopamine neuron destruction occuring at a faster rate than neuroplasticity. This might result in the larger lesion created masking the beneficial effects of exercise in neurotrophic factor expression.

8.5 CONCLUSION

The prevalence of psychological disorders such as depression and anxiety in adults that were exposed to various forms of stress in childhood, has resulted in the maternal separation rat models being one of the preferred types of animal models used to investigate these disorders. These models as discussed in *Section 1.8,* have a tendency to respond to stressors by elevating circulating corticosterones that have been shown to have an inhibitory effect on the secretion of neurotrophic factors. Neurotrophic factor expression has been shown to be maximal during brain development and gradually decreases with age. The diminished neurotrophic factor level with age has been suggested as

one of the reasons for progressive neurodegeneration in Parkinson's disease. Therefore using a model that has been shown to respond to stress by increasing corticosterone which has been shown to be inhibitory to neurotrophic factor expression as a Parkinsonian rat model, might exacerbate the neurotoxic effect of 6-OHDA in the substantia nigra. In the present study we have shown that exercise did not alter motor function impairments that might be due to the altered neuronal circuits present in some models of maternal separation but decreased the amount of dopamine neuron destruction following 6-OHDA infusion. This suggests that exercise provides neuroprotection in adult rats that have been maternally separated. Therefore exposure of rats to early life stressors does not predispose them to greater dopamine neurodegeneration than non-stressed rats in a Parkinsonian rat model. This might suggest that individuals who were exposed to emotional trauma in early childhood do not have a greater predisposition to Parkinson's disease than less exposed individuals.

CHAPTER 9

SUMMARY

Studies that have demonstrated neuroprotection in a Parkinsonian rat model relied mostly on forcing the rat to exercise the injured forelimb post unilateral 6-OHDA injection *(Tillerson et al 2001).* Other studies that also examined neuroprotection following a brain insult forced the rats to exercise on a treadmill *(Carro et al 2001).* In our study the fact that rats in the immobilised wheels did have access to the wheel but could not run raises a question of whether there is an optimum amount of exercise that a rat should have in order to protect the dopamine neurons. In addition to neuroprotection there is also evidence of adaptation such as the decrease in non-lesioned hemisphere striatal dopamine which accounts for the absence of asymmetrical behaviour associated with apomorphine injection in unilaterally 6-OHDA infused rats that had access to running wheels. Exposure to free running wheels results in greater dopamine neuron sparing as shown by dopamine neuron destruction in the substantia nigra of the lesioned hemisphere. Therefore exposure to free running wheels appears to be important as a neuroprotective effect in rats that have been lesioned to mimic Parkinson's disease.

Neuroprotection in adult rats is due to increased expression of neurotrophic factors. These neurotrophic factors are peptides that are found inside and outside the brain and have been shown to be increased follolowing injury to the the neurons. Studies focusing on neurodegenerative diseases of the basal ganglia such as Parkinson's diseases have identified GDNF which is a member of the GDNF superfamily of neurotrophic factors as playing a key role in increasing midbrain dopamine levels, dopamine neuron protection from neurotoxins, and in the maintenance of injured dopamine cell. Exercise has been shown to increase the expression of intra brain and extra brain neurotrophic

factors and thus providing neuroprotection to dopamine neurons following injury. In our investigation into the effects of exercise on GDNF expression in rats that were lesioned with 6-OHDA, we found that GNDF expression is not increased 14 days after the lesion. If GDNF provides neuroprotection in exercising rats then GDNF surges are transient and decrease soon after the infusion of neurotoxins as suggested by *Cohen et al (2003).* This is supported by the fact that in rats that started exercising after the GDNF surge had passed, there was complete destruction of dopamine neurons in the nigrostriatal pathway *(Tillerson et al 2001). Howells et al (2005)* has also shown that following apomorphine injection into 6-OHDA infused rats, there were significantly more apomorphine-induced turns made by the stressed rats that exercised than in non-stressed rats with access to running wheels suggesting that stress cancelled the benefical effects of exercise. An increase in basal corticosterone levels in 6-OHDA infused rats that had access to running wheels does not seem to exarcebate dopamine neuron destruction or GDNF concentration but the addition of exogenous stressors results in a decrease in GDNF concentration in the substantia nigra of stressed rats. As Parkinson's disease is a progressive neurodegenerative disease prescribing a treatment protocol that involves moderate exercise and a reduction in exposure to stress might slow down the progression of the neurodegeneration.

Studies have also shown that GDNF expression is maximal early in life when the neuronal circuitry is still forming *(Stromberg et al 1993).* In vitro studies have shown that GDNF expression is associated with an increase in dopamine neuron size and in the number of axonal and dendritic process *(Lopes-Martin et al 1999)* suggesting that the sturdiness of the neurons can be crucial in withstanding the toxic effects of 6-OHDA. However GDNF expression rapidly decreases as development proceeds *(Stromberg et al 1993)* and increases in GDNF expression in adult rats occur when there is injury to the neurons *(Naveilhan et al 1997). Cohen et al (2003),* has shown that GDNF expression is increased in the lesioned hemisphere of exercising rats. We found that in the absence of

exogenous stressors, exercise provides neuroprotection following 6-OHDA infusion in the MFB. We also found that ACTH and corticosterone levels in plasma were not significantly increased after acute restraint stress. However the rats were not lesioned and hence would not have been as stressed as the 6-OHDA lesioned rats previously mentioned. It has also been suggested that Sprague Dawley rats are diurnally inactive *(Schallert et al 2000)* suggesting that as the rats were taken out of their cages with attached running wheels during their light cycle, the need for energy mobilisation by increasing corticosterone levels would have been low. The absence of a corticosterone response to restraint stress in exercised rats might suggest that the neuronal circuitry of the HPA axis adapts to the stress of exercise by marginal desensitisation of the CRF receptors in the pituitary gland thus limiting the secretion of ACTH during transient increases in stress. The absence of an increased GDNF expression in rats with access to running wheels suggests that GDNF changes after the perinatal period only occur in the presence of brain trauma. Increase in GDNF expression has also been shown to be transient even in the presence of brain injury suggesting that an increase in GDNF expression is unlikely to be seen 3 weeks after exercise was started. Therefore the beneficial effect of exercise in exercised non-lesioned rats seems to be the increased threshold for ACTH and thus corticosterone release. As GDNF has been shown to be negatively correlated to corticosterone levels, exercise pre and post lesion might be beneficial in negating corticosterone surges during and after 6-OHDA lesion. Having established that the addition of exogenous stressors in adult rats cancels the beneficial effect of exercise, we looked at whether perinatal models of stress have an increased vulnerability to the toxic effect of 6-OHDA and whether exercise can provide neuroprotection in adult exercised rats that were prenatally stressed or maternally separated (first two weeks after birth). In prenatal stress models, the duration and type of stressor used determines the adult offspring's response to an acute stressor. We wanted to create a mild prenatal stress model that did not have the increased basal corticosterone levels or increased anxiety-like behaviour associated with many prenatal stress models. The 50% food

deprivation model proposed in this study was of acute duration (6 days) and did not produce any differences in the size of the adrenal glands, plasma corticosterone levels or the ACTH response to restraint stress. The duration of the early life stressor plays a role in the rat's response to stress since the 75% food deprivation model (Ježová *et al.*, 2002) was of similar duration as the present study and yielded similar results. The mild stressor model described in the present study differed from other mild stressor models in that it produced normal adrenal glands and normal basal plasma corticosterone and ACTH levels. This model also displayed less locomotor activity than non-stressed rats in the open field and a slightly blunted plasma ACTH response following acute restraint. It is possible that this could be a viable prenatal stress model to study subtle changes in HPA axis activity and its effects on different areas of the brain including the limbic system and basal ganglia. However it is important to note that other factors including handling and the environment may influence the development of the HPA axis and thus its response to stressors in adult offspring.

By using small doses of 6-OHDA, we were able to create dopamine neuron destruction more representative of early Parkinson's disease *(Truong et al 2006).* This made it possible to unmask the beneficial effects of exercise in non-stressed rats. In a prenatal stress rat model, injecting a small dose of 6-OHDA resulted in a lesion more consistent with larger doses of 6-OHDA *(Truong et al 2006)* with dopamine neuron destruction equivalent to the destruction seen when a higher dose of 6-OHDA was used in exercised rats, thus implying that the prenatally stressed rats are more vulnerable to the toxic effects of 6-OHDA than non-stressed rat. Therefore trauma to the substantia nigra might increase the susceptibility to developing Parkinson's disease in people or animals that were exposed to prenatal stress in utero.

Studies looking at maternal separation models of stress have mostly investigated the effects of maternal separation on adult rats. We wanted to investigate whether juvenile rats that were maternally separated exhibited the same response to stress as adult rats that were maternally separated. We found that

there was a blunted ACTH response to acute restraint stress in 7 week-old rats that were maternally separated and did not exercise. This response was similar to that shown by *Daniels et al (2004)* in adult rats that were maternally separated rats. However exercise reversed the ACTH response to acute restraint stress in these rats. Studies using the maternal separation model to study the effects of childhood trauma have mainly focused on psychosomatic and affective disorders such as depression. Studies have shown that these disorders can have a neurodegenerative aspect to them as suggested by the decrease in the size of the affected areas such as the hippocampus. This suggests that the abnormal HPA axis has a role in causing progressive neurodegeneration in the hippocampus. This raises the question of whether maternal separation affects other brain areas and may thus lead to an increased vulnerability to neurodegenerative diseases such as Parkinson's disease. In lesioned adult offspring of prenatally stressed rats, we found that, prenatally stressed rats that have also been shown to have an abnormal ACTH response to acute restraint stress were more vulnerable to the toxic insult of 6-OHDA which was not reversed by exercise. Therefore a mild early postnatal stress model such as the one used in the present study might be valuable in investigating whether perinatal stressors result in increased vulnerability to 6-OHDA infusion in a rat model for Parkinson's disease which can have implications in the ongoing investigations into the aetiology of Parkinson's disease.

The prevalence of psychological disorders such as depression and anxiety in adults that were exposed to various forms of stress in childhood, has resulted in the maternal separation rat models being one of the preferred types of animal models used to investigate these disorders. Adult rats that were maternally separated , have a tendency to respond to stressors by elevating circulating corticosterones that have been shown to have an inhibitory effect on the secretion of neurotrophic factors. Neurotrophic factor expression has been shown to be maximal during brain development and gradually decreases with age. The diminished neurotrophic factor level with age has been suggested as

one of the reasons for progressive neurodegeneration in Parkinson's disease. Therefore using a model that has been shown to respond to stress by increasing corticosterone which has been shown to be inhibitory to neurotrophic factor expression in a Parkinsonian rat model, might exacerbate the neurotoxic effect of 6-OHDA in the substantia nigra. In the present study we have shown that exercise did not alter motor function impairments that might be due to the altered neuronal circuits present in some models of maternal separation but decreased the amount of dopamine neuron destruction following 6-OHDA infusion. This suggests that exercise provides neuroprotection in adult rats that have been maternally separated. Therefore exposure of rats to early life stressors does not predispose them to greater dopamine neurodegeneration than non-stressed rats in a Parkinsonian rat model. This might suggest that individuals who were exposed to emotional trauma in early childhood do not have a greater predisposition to Parkinson's disease than less exposed individuals.

In conclusion voluntary exercise provides neuroprotection in 6-OHDA lesioned rats but the beneficial effects of exercise are cancelled by the introduction of exogenous stressors immediately following lesion. GDNF expression was not increased in lesioned and non-lesioned rats that were exercised 21 day after 6-OHDA lesioning or the commencement of the exercise protoctol. However exercise in non-lesioned rats decreased the corticosterone response to acute restraint stress. In a mild prenatal stress model, adult offspring that were lesioned with a small dose of 6-OHDA did not show neuroprotection following exercise and in non-exercised rats the dopamine neuron destruction was as severe as that seen when a large dose was used. However in adult maternally separated rats that were exercised, dopamine neuron destruction was similar to that seen in non-stressed exercised rats lesioned with a similar dose of 6-OHDA suggesting that exercise provided neuroprotection in maternally separated rats. This suggests that the dopamine neurons in the nigrostriatal pathway of adult offspring of rats that were prenatally stressed are more vulnerable to the toxic effects of 6-OHDA than adult rats that were maternally separated.

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	Parameter	A Value
		Y
1	Table Analyzed	CHAPTER 2, APOMORPH
2	Column A	RUNNERS
3	vs	vs
4	Column B	NON-RUNNERS
5		
6	Unpaired t test	
7	P value	0.0001
8	P value summary	***
9	Are means signif. different? (P < 0.05)	Yes
10	One- or two-tailed P value?	Two-tailed
11	t, df	t=14.11 df=4
12		
13	How big is the difference?	
14	Mean ± SEM of column A	-15.89 ± 6.052 N=3
15	Mean ± SEM of column B	152.6 ± 10.29 N=3
16	Difference between means	-168.5 ± 11.94
17	95% confidence interval	-201.6 to -135.3
18	R squared	0.9803
19		
20	F test to compare variances	
21	F,DFn, Dfd	2.893, 2, 2
22	P value	0.5138
23	P value summary	ns
24	Are variances significantly different?	No

	Parameter	A Value
		Y
1	Table Analyzed	CHAPTER 2, TYROSINE HYDROXYL
2	Column A	R
3	vs	vs
4	Column B	NR
5		
6	Unpaired t test	
7	P value	0.0061
8	P value summary	**
9	Are means signif. different? (P < 0.05)	Yes
10	One- or two-tailed P value?	Two-tailed
11	t, df	t=2.999 df=25
12		
13	How big is the difference?	
14	Mean ± SEM of column A	64.89 ± 2.800 N=14
15	Mean ± SEM of column B	86.15 ± 6.720 N=13
16	Difference between means	-21.26 ± 7.090
17	95% confidence interval	-35.87 to -6.654
18	R squared	0.2645
19		
20	F test to compare variances	
21	F,DFn, Dfd	5.349, 12, 13
22	P value	0.0052
23	P value summary	**
24	Are variances significantly different?	Yes

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 2, STRIATUM DOPAMINE				
3	One-way analysis of variance				
4	P value	0.0019			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	6.189			
9	R squared	0.3672			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	3.290			
13	P value	0.3490			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	39830000	3	13280000	
19	Residual (within columns)	68640000	32	2145000	
20	Total	108500000	35		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	DA non-lesion (run) vs DA lesion (run)	497.9	1.020	P > 0.05	-1374 to 2370
24	DA non-lesion (run) vs DA non-lesion (nor	-1909	3.911	P < 0.05	-3781 to -37.47
25	DA non-lesion (run) vs DA lesion (non-run	792.3	1.623	P > 0.05	-1079 to 2664
26	DA lesion (run) vs DA non-lesion (non-run	-2407	4.931	P < 0.01	-4279 to -535.3
27	DA lesion (run) vs DA lesion (non-run)	294.4	0.6031	P > 0.05	-1577 to 2166
28	DA non-lesion (non-run) vs DA lesion (nor	2702	5.534	P < 0.01	829.8 to 4573

	Parameter	A Value
		Y
1	Table Analyzed	CHAPTER 2, % DOPAMI
2	Column A	R
3	vs	vs
4	Column B	NR
5		
6	Unpaired t test	
7	P value	0.0002
8	P value summary	***
9	Are means signif. different? (P < 0.05)	Yes
10	One- or two-tailed P value?	Two-tailed
11	t, df	t=4.699 df=16
12		
13	How big is the difference?	
14	Mean ± SEM of column A	74.60 ± 6.000 N=9
15	Mean ± SEM of column B	30.20 ± 7.300 N=9
16	Difference between means	44.40 ± 9.449
17	95% confidence interval	24.37 to 64.43
18	R squared	0.5798
19		
20	F test to compare variances	
21	F,DFn, Dfd	1.480, 8, 8
22	P value	0.5919
23	P value summary	ns
24	Are variances significantly different?	No

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 2, DOPAC CONCENTRATION				
3	One-way analysis of variance				
4	P value	0.1178			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	4			
8	F	2.115			
9	R squared	0.1655			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	0.9172			
13	P value	0.8213			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	442700	3	147600	
19	Residual (within columns)	2233000	32	69770	
20	Total	2675000	35		
21					
22	No post tests. P > 0.05				

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 2, % DOPAC REMAINING				
3	One-way analysis of variance				
4	P value	0.1178			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	4			
8	F	2.115			
9	R squared	0.1655			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	0.9172			
13	P value	0.8213			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	442700	3	147600	
19	Residual (within columns)	2233000	32	69770	
20	Total	2675000	35		
21					
22	No post tests. P > 0.05				

	Parameter	A Value
		Y
1	Table Analyzed	CHAPTER 2, % DOPAMI
2	Column A	R
3	vs	vs
4	Column B	NR
5		
6	Unpaired t test	
7	P value	0.0002
8	P value summary	***
9	Are means signif. different? (P < 0.05)	Yes
10	One- or two-tailed P value?	Two-tailed
11	t, df	t=4.699 df=16
12		
13	How big is the difference?	
14	Mean ± SEM of column A	74.60 ± 6.000 N=9
15	Mean ± SEM of column B	30.20 ± 7.300 N=9
16	Difference between means	44.40 ± 9.449
17	95% confidence interval	24.37 to 64.43
18	R squared	0.5798
19		
20	F test to compare variances	
21	F,DFn, Dfd	1.480, 8, 8
22	P value	0.5919
23	P value summary	ns
24	Are variances significantly different?	No

		A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 2, DOPAC CONCENTRATION				
3	One-way analysis of variance				
4	P value	0.1178			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	4			
8	F	2.115			
9	R squared	0.1655			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	0.9172			
13	P value	0.8213			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	442700	3	147600	
19	Residual (within columns)	2233000	32	69770	
20	Total	2675000	35		
21					
22	No post tests. P > 0.05				

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 2, % DOPAC REMAINING				
3	One-way analysis of variance				
4	P value	0.1178			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	4			
8	F	2.115			
9	R squared	0.1655			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	0.9172			
13	P value	0.8213			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	442700	3	147600	
19	Residual (within columns)	2233000	32	69770	
20	Total	2675000	35		
21					
22	No post tests. P > 0.05				

	Parameter	A
		Value
		Y
1	Table Analyzed	CHAPTER 3, ACTH
2	Column A	NR
3	vs	vs
4	Column B	R
5		
6	Unpaired t test	
7	P value	0.0463
8	P value summary	*
9	Are means signif. different? (P < 0.05)	Yes
10	One- or two-tailed P value?	Two-tailed
11	t, df	t=2.107 df=23
12		
13	How big is the difference?	
14	Mean ± SEM of column A	83.64 ± 9.785 N=14
15	Mean ± SEM of column B	125.4 ± 18.62 N=11
16	Difference between means	-41.72 ± 19.80
17	95% confidence interval	-82.70 to -0.7459
18	R squared	0.1617
19		
20	F test to compare variances	
21	F,DFn, Dfd	2.846, 10, 13
22	P value	0.0806
23	P value summary	ns
24	Are variances significantly different?	No

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 3, CORT				
3	One-way analysis of variance				
4	P value	0.0061			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	3			
8	F	6.077			
9	R squared	0.2883			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	10.67			
13	P value	0.0048			
14	P value summary	**			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	330500	2	165300	
19	Residual (within columns)	815800	30	27190	
20	Total	1146000	32		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NR vs R	-229.7	4.543	P < 0.01	-406.0 to -53.44
24	NR vs SR	-176.5	3.694	P < 0.05	-343.0 to -9.940
25	R vs SR	53.23	1.016	P > 0.05	-129.5 to 235.9

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 3, STRIATUM				
3	One-way analysis of variance				
4	P value	0.6029			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	6			
8	F	0.7308			
9	R squared	0.05246			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	36.04			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	9914	5	1983	
19	Residual (within columns)	179100	66	2713	
20	Total	189000	71		
21					
22	No post tests. P > 0.05				

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 3, SN				
3	One-way analysis of variance				
4	P value	0.0065			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	6			
8	F	3.560			
9	R squared	0.2124			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	6.768			
13	P value	0.2385			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	311000	5	62200	
19	Residual (within columns)	1153000	66	17470	
20	Total	1464000	71		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NR L vs R L	65.86	1.749	P > 0.05	-90.68 to 222.4
24	NR L vs SR L	80.21	2.130	P > 0.05	-76.33 to 236.7
25	NR L vs NR R	-12.95	0.3665	P > 0.05	-159.8 to 133.9
26	NR L vs R R	134.0	3.558	P > 0.05	-22.52 to 290.5
27	NR L vs SR R	165.9	4.404	P < 0.05	9.320 to 322.4
28	R L vs SR L	14.35	0.3600	P > 0.05	-151.3 to 180.0
29	R L vs NR R	-78.81	2.092	P > 0.05	-235.3 to 77.73
30	R L vs R R	68.16	1.710	P > 0.05	-97.50 to 233.8
31	R L vs SR R	100.0	2.509	P > 0.05	-65.66 to 265.7
32	SR L vs NR R	-93.16	2.473	P > 0.05	-249.7 to 63.38
33	SR L vs R R	53.81	1.350	P > 0.05	-111.9 to 219.5
34	SR L vs SR R	85.65	2.149	P > 0.05	-80.01 to 251.3
35	NR R vs R R	147.0	3.902	P > 0.05	-9.571 to 303.5
36	NR R vs SR R	178.8	4.748	P < 0.05	22.27 to 335.3
37	R R vs SR R	31.84	0.7988	P > 0.05	-133.8 to 197.5

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 3, VTA				
3	One-way analysis of variance				
4	P value	0.6452			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	6			
8	F	0.6732			
9	R squared	0.04852			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	20.83			
13	P value	0.0009			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	200900	5	40190	
19	Residual (within columns)	3940000	66	59700	
20	Total	4141000	71		
21					
22	No post tests. P > 0.05				

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 4, ACTH				
3	One-way analysis of variance				
4	P value	0.2223			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	6			
8	F	1.470			
9	R squared	0.1621			
10					
11	ANOVA Table	SS	df	MS	
12	Treatment (between columns)	32690	5	6538	
13	Residual (within columns)	169000	38	4447	
14	Total	201700	43		
15					
16	No post tests. P > 0.05				

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 4, CORT				
3	One-way analysis of variance				
4	P value	0.0009			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	6			
8	F	5.277			
9	R squared	0.4098			
10					
11	ANOVA Table	SS	df	MS	
12	Treatment (between columns)	775300	5	155100	
13	Residual (within columns)	1117000	38	29390	
14	Total	1892000	43		
15					
16	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
17	NR (BASAL) vs R (BASAL)	80.44	1.408	P > 0.05	-162.2 to 323.1
18	NR (BASAL) vs NR (15 MIN)	-303.0	5.145	P < 0.01	-553.1 to -52.95
19	NR (BASAL) vs R (15 MIN)	-151.3	2.716	P > 0.05	-387.7 to 85.22
20	NR (BASAL) vs NR (1H)	-38.67	0.5308	P > 0.05	-348.0 to 270.6
21	NR (BASAL) vs R (1H)	8.333	0.1144	P > 0.05	-301.0 to 317.6
22	R (BASAL) vs NR (15 MIN)	-383.5	6.511	P < 0.001	-633.6 to -133.4
23	R (BASAL) vs R (15 MIN)	-231.7	4.160	P > 0.05	-468.2 to 4.772
24	R (BASAL) vs NR (1H)	-119.1	1.635	P > 0.05	-428.4 to 190.2
25	R (BASAL) vs R (1H)	-72.11	0.9900	P > 0.05	-381.4 to 237.2
26	NR (15 MIN) vs R (15 MIN)	151.8	2.640	P > 0.05	-92.36 to 395.9
27	NR (15 MIN) vs NR (1H)	264.4	3.562	P > 0.05	-50.81 to 579.6
28	NR (15 MIN) vs R (1H)	311.4	4.195	P > 0.05	-3.806 to 626.6
29	R (15 MIN) vs NR (1H)	112.6	1.570	P > 0.05	-191.9 to 417.1
30	R (15 MIN) vs R (1H)	159.6	2.226	P > 0.05	-144.9 to 464.1
31	NR (1H) vs R (1H)	47.00	0.5483	P > 0.05	-316.9 to 410.9

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 4, GDNF, LEFT HEM				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	6			
8	F	11.80			
9	R squared	0.3189			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	110.6			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	1475000	5	294900	
19	Residual (within columns)	3150000	126	25000	
20	Total	4624000	131		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NR STRI (L) vs NR SN (L)	-182.0	5.275	P < 0.01	-323.3 to -40.69
24	NR STRI (L) vs NR VTA (L)	-285.0	8.260	P < 0.001	-426.3 to -143.7
25	NR STRI (L) vs L STRI (R)	-1.000	0.02964	P > 0.05	-139.2 to 137.2
26	NR STRI (L) vs L SN (R)	-119.0	3.527	P > 0.05	-257.2 to 19.21
27	NR STRI (L) vs L VTA (R)	-217.0	6.431	P < 0.001	-355.2 to -78.79
28	NR SN (L) vs NR VTA (L)	-103.0	2.985	P > 0.05	-244.3 to 38.31
29	NR SN (L) vs L STRI (R)	181.0	5.364	P < 0.01	42.79 to 319.2
30	NR SN (L) vs L SN (R)	63.00	1.867	P > 0.05	-75.21 to 201.2
31	NR SN (L) vs L VTA (R)	-35.00	1.037	P > 0.05	-173.2 to 103.2
32	NR VTA (L) vs L STRI (R)	284.0	8.416	P < 0.001	145.8 to 422.2
33	NR VTA (L) vs L SN (R)	166.0	4.919	P < 0.01	27.79 to 304.2
34	NR VTA (L) vs L VTA (R)	68.00	2.015	P > 0.05	-70.21 to 206.2
35	L STRI (R) vs L SN (R)	-118.0	3.579	P > 0.05	-253.0 to 17.03
36	L STRI (R) vs L VTA (R)	-216.0	6.552	P < 0.001	-351.0 to -80.97
37	L SN (R) vs L VTA (R)	-98.00	2.973	P > 0.05	-233.0 to 37.03

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 4, GDNF, RIGHT HEM				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	6			
8	F	16.27			
9	R squared	0.3923			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	114.8			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	1561000	5	312100	
19	Residual (within columns)	2417000	126	19190	
20	Total	3978000	131		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NR STRI vs NR SN (L)	-211.0	6.981	P < 0.001	-334.8 to -87.20
24	NR STRI vs NR VTA (L)	-287.0	9.495	P < 0.001	-410.8 to -163.2
25	NR STRI vs R STRI (R)	4.000	0.1353	P > 0.05	-117.1 to 125.1
26	NR STRI vs R SN (R)	-121.0	4.093	P > 0.05	-242.1 to 0.08009
27	NR STRI vs R VTA (R)	-208.0	7.036	P < 0.001	-329.1 to -86.92
28	NR SN (L) vs NR VTA (L)	-76.00	2.514	P > 0.05	-199.8 to 47.80
29	NR SN (L) vs R STRI (R)	215.0	7.273	P < 0.001	93.92 to 336.1
30	NR SN (L) vs R SN (R)	90.00	3.044	P > 0.05	-31.08 to 211.1
31	NR SN (L) vs R VTA (R)	3.000	0.1015	P > 0.05	-118.1 to 124.1
32	NR VTA (L) vs R STRI (R)	291.0	9.844	P < 0.001	169.9 to 412.1
33	NR VTA (L) vs R SN (R)	166.0	5.615	P < 0.01	44.92 to 287.1
34	NR VTA (L) vs R VTA (R)	79.00	2.672	P > 0.05	-42.08 to 200.1
35	R STRI (R) vs R SN (R)	-125.0	4.328	P < 0.05	-243.3 to -6.704
36	R STRI (R) vs R VTA (R)	-212.0	7.340	P < 0.001	-330.3 to -93.70
37	R SN (R) vs R VTA (R)	-87.00	3.012	P > 0.05	-205.3 to 31.30

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 5, ELEVATED + MAZE				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	6			
8	F	39.81			
9	R squared	0.6483			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	3.576			
13	P value	0.6118			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	266900	5	53380	
19	Residual (within columns)	144800	108	1341	
20	Total	411700	113		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NON-STRESSED (OPEN) vs MILDLY ST	-1.295	0.1521	P > 0.05	-36.29 to 33.70
24	NON-STRESSED (OPEN) vs Column C	13.49	1.604	P > 0.05	-21.08 to 48.06
25	NON-STRESSED (OPEN) vs NON-STRE	-80.30	9.304	P < 0.001	-115.8 to -44.83
26	NON-STRESSED (OPEN) vs MILDLY ST	-92.72	10.89	P < 0.001	-127.7 to -57.72
27	NON-STRESSED (OPEN) vs FOOD-DEF	-99.91	11.88	P < 0.001	-134.5 to -65.34
28	MILDLY STRESSED (OPEN) vs Column	14.78	1.782	P > 0.05	-19.30 to 48.87
29	MILDLY STRESSED (OPEN) vs NON-ST	-79.00	9.277	P < 0.001	-114.0 to -44.01
30	MILDLY STRESSED (OPEN) vs MILDLY	-91.42	10.88	P < 0.001	-125.9 to -56.90
31	MILDLY STRESSED (OPEN) vs FOOD-D	-98.62	11.89	P < 0.001	-132.7 to -64.53
32	Column C vs NON-STRESSED (CLOSED	-93.79	11.15	P < 0.001	-128.4 to -59.22
33	Column C vs MILDLY STRESSED (CLOS	-106.2	12.80	P < 0.001	-140.3 to -72.12
34	Column C vs FOOD-DEPRIVED (CLOSE	-113.4	13.85	P < 0.001	-147.0 to -79.75
35	NON-STRESSED (CLOSED) vs MILDLY	-12.42	1.458	P > 0.05	-47.41 to 22.58
36	NON-STRESSED (CLOSED) vs FOOD-D	-19.61	2.331	P > 0.05	-54.18 to 14.96
37	MILDLY STRESSED (CLOSED) vs FOOD	-7.195	0.8674	P > 0.05	-41.28 to 26.89

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 5, OPEN FIELD				
3	One-way analysis of variance				
4	P value	0.0221			
5	P value summary	*			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	3			
8	F	4.126			
9	R squared	0.1441			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	0.2151			
13	P value	0.8980			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	2919000	2	1459000	
19	Residual (within columns)	17330000	49	353700	
20	Total	20250000	51		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Non-stressed vs Stressed	577.0	4.057	P < 0.05	90.24 to 1064
24	Non-stressed vs Food-deprived	254.0	1.786	P > 0.05	-232.8 to 740.8
25	Stressed vs Food-deprived	-323.0	2.239	P > 0.05	-816.7 to 170.7

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 5, ADRENAL WEIGHTS				
3	One-way analysis of variance				
4	P value	0.8385			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	3			
8	F	0.1768			
9	R squared	0.006884			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	1.845			
13	P value	0.3975			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	0.0003360	2	0.0001680	
19	Residual (within columns)	0.04847	51	0.0009504	
20	Total	0.04881	53		
21					
22	No post tests. P > 0.05				

	Parameter	A	B	C	D
		Value	Data Set-B	Data Set-C	Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 5, CORT				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	9			
8	F	13.50			
9	R squared	0.7059			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	8.053			
13	P value	0.4283			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	1018000	8	127200	
19	Residual (within columns)	424000	45	9423	
20	Total	1442000	53		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	non-stressed (0 MIN) vs stressed (0 MIN)	-51.00	1.287	P > 0.05	-233.8 to 131.8
24	non-stressed (0 MIN) vs food-deprived (0	-9.000	0.2271	P > 0.05	-191.8 to 173.8
25	non-stressed (0 MIN) vs non-stressed (15	-329.0	8.302	P < 0.001	-511.8 to -146.2
26	non-stressed (0 MIN) vs stressed (15 MIN	-350.0	8.832	P < 0.001	-532.8 to -167.2
27	non-stressed (0 MIN) vs food-deprived (1	-336.0	8.479	P < 0.001	-518.8 to -153.2
28	non-stressed (0 MIN) vs non-stressed (30	-260.0	6.561	P < 0.001	-442.8 to -77.16
29	non-stressed (0 MIN) vs stressed (30 MIN	-252.0	6.359	P < 0.01	-434.8 to -69.16
30	non-stressed (0 MIN) vs food-deprived (0	-287.0	7.242	P < 0.001	-469.8 to -104.2
31	stressed (0 MIN) vs food-deprived (0 MIN	42.00	1.060	P > 0.05	-140.8 to 224.8
32	stressed (0 MIN) vs non-stressed (15 MIN	-278.0	7.015	P < 0.001	-460.8 to -95.16
33	stressed (0 MIN) vs stressed (15 MIN)	-299.0	7.545	P < 0.001	-481.8 to -116.2
34	stressed (0 MIN) vs food-deprived (15 MI	-285.0	7.192	P < 0.001	-467.8 to -102.2
35	stressed (0 MIN) vs non-stressed (30 MIN	-209.0	5.274	P < 0.05	-391.8 to -26.16
36	stressed (0 MIN) vs stressed (30 MIN)	-201.0	5.072	P < 0.05	-383.8 to -18.16
37	stressed (0 MIN) vs food-deprived (0 MIN	-236.0	5.955	P < 0.01	-418.8 to -53.16
38	food-deprived (0 MIN) vs non-stressed (1	-320.0	8.075	P < 0.001	-502.8 to -137.2
39	food-deprived (0 MIN) vs stressed (15 MI	-341.0	8.605	P < 0.001	-523.8 to -158.2
40	food-deprived (0 MIN) vs food-deprived (1	-327.0	8.252	P < 0.001	-509.8 to -144.2
41	food-deprived (0 MIN) vs non-stressed (3	-251.0	6.334	P < 0.01	-433.8 to -68.16
42	food-deprived (0 MIN) vs stressed (30 MI	-243.0	6.132	P < 0.01	-425.8 to -60.16
43	food-deprived (0 MIN) vs food-deprived (0	-278.0	7.015	P < 0.001	-460.8 to -95.16
44	non-stressed (15 MIN) vs stressed (15 MI	-21.00	0.5299	P > 0.05	-203.8 to 161.8

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
45	non-stressed (15 MIN) vs food-deprived (	-7.000	0.1766	P > 0.05	-189.8 to 175.8
46	non-stressed (15 MIN) vs non-stressed (3	69.00	1.741	P > 0.05	-113.8 to 251.8
47	non-stressed (15 MIN) vs stressed (30 MI	77.00	1.943	P > 0.05	-105.8 to 259.8
48	non-stressed (15 MIN) vs food-deprived (	42.00	1.060	P > 0.05	-140.8 to 224.8
49	stressed (15 MIN) vs food-deprived (15 M	14.00	0.3533	P > 0.05	-168.8 to 196.8
50	stressed (15 MIN) vs non-stressed (30 MI	90.00	2.271	P > 0.05	-92.84 to 272.8
51	stressed (15 MIN) vs stressed (30 MIN)	98.00	2.473	P > 0.05	-84.84 to 280.8
52	stressed (15 MIN) vs food-deprived (0 MI	63.00	1.590	P > 0.05	-119.8 to 245.8
53	food-deprived (15 MIN) vs non-stressed (	76.00	1.918	P > 0.05	-106.8 to 258.8
54	food-deprived (15 MIN) vs stressed (30 M	84.00	2.120	P > 0.05	-98.84 to 266.8
55	food-deprived (15 MIN) vs food-deprived (	49.00	1.236	P > 0.05	-133.8 to 231.8
56	non-stressed (30 MIN) vs stressed (30 MI	8.000	0.2019	P > 0.05	-174.8 to 190.8
57	non-stressed (30 MIN) vs food-deprived (	-27.00	0.6813	P > 0.05	-209.8 to 155.8
58	stressed (30 MIN) vs food-deprived (0 MI	-35.00	0.8832	P > 0.05	-217.8 to 147.8

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 5, ACTH				
3	One-way analysis of variance				
4	P value	0.0012			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	9			
8	F	3.987			
9	R squared	0.4148			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	20.92			
13	P value	0.0074			
14	P value summary	**			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	49100	8	6138	
19	Residual (within columns)	69280	45	1539	
20	Total	118400	53		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	non-stressed (0 MIN) vs stressed (0 MIN)	0.0000	0.0000	P > 0.05	-73.90 to 73.90
24	non-stressed (0 MIN) vs food-deprived (0	-2.000	0.1249	P > 0.05	-75.90 to 71.90
25	non-stressed (0 MIN) vs non-stressed (15	-79.00	4.932	P < 0.05	-152.9 to -5.097
26	non-stressed (0 MIN) vs stressed (15 MIN	-63.00	3.933	P > 0.05	-136.9 to 10.90
27	non-stressed (0 MIN) vs food-deprived (15	-63.00	3.933	P > 0.05	-136.9 to 10.90
28	non-stressed (0 MIN) vs non-stressed (30	-51.00	3.184	P > 0.05	-124.9 to 22.90
29	non-stressed (0 MIN) vs stressed (30 MIN	-68.00	4.245	P > 0.05	-141.9 to 5.903
30	non-stressed (0 MIN) vs food-deprived (0	-49.00	3.059	P > 0.05	-122.9 to 24.90
31	stressed (0 MIN) vs food-deprived (0 MIN	-2.000	0.1249	P > 0.05	-75.90 to 71.90
32	stressed (0 MIN) vs non-stressed (15 MIN	-79.00	4.932	P < 0.05	-152.9 to -5.097
33	stressed (0 MIN) vs stressed (15 MIN)	-63.00	3.933	P > 0.05	-136.9 to 10.90
34	stressed (0 MIN) vs food-deprived (15 MI	-63.00	3.933	P > 0.05	-136.9 to 10.90
35	stressed (0 MIN) vs non-stressed (30 MIN	-51.00	3.184	P > 0.05	-124.9 to 22.90
36	stressed (0 MIN) vs stressed (30 MIN)	-68.00	4.245	P > 0.05	-141.9 to 5.903
37	stressed (0 MIN) vs food-deprived (0 MIN	-49.00	3.059	P > 0.05	-122.9 to 24.90
38	food-deprived (0 MIN) vs non-stressed (15	-77.00	4.807	P < 0.05	-150.9 to -3.097
39	food-deprived (0 MIN) vs stressed (15 MI	-61.00	3.808	P > 0.05	-134.9 to 12.90
40	food-deprived (0 MIN) vs food-deprived (1	-61.00	3.808	P > 0.05	-134.9 to 12.90
41	food-deprived (0 MIN) vs non-stressed (3	-49.00	3.059	P > 0.05	-122.9 to 24.90
42	food-deprived (0 MIN) vs stressed (30 MI	-66.00	4.120	P > 0.05	-139.9 to 7.903
43	food-deprived (0 MIN) vs food-deprived (0	-47.00	2.934	P > 0.05	-120.9 to 26.90
44	non-stressed (15 MIN) vs stressed (15 MI	16.00	0.9989	P > 0.05	-57.90 to 89.90

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
45	non-stressed (15 MIN) vs food-deprived (	16.00	0.9989	P > 0.05	-57.90 to 89.90
46	non-stressed (15 MIN) vs non-stressed (3	28.00	1.748	P > 0.05	-45.90 to 101.9
47	non-stressed (15 MIN) vs stressed (30 MI	11.00	0.6867	P > 0.05	-62.90 to 84.90
48	non-stressed (15 MIN) vs food-deprived (	30.00	1.873	P > 0.05	-43.90 to 103.9
49	stressed (15 MIN) vs food-deprived (15 M	0.0000	0.0000	P > 0.05	-73.90 to 73.90
50	stressed (15 MIN) vs non-stressed (30 MI	12.00	0.7492	P > 0.05	-61.90 to 85.90
51	stressed (15 MIN) vs stressed (30 MIN)	-5.000	0.3121	P > 0.05	-78.90 to 68.90
52	stressed (15 MIN) vs food-deprived (0 MI	14.00	0.8740	P > 0.05	-59.90 to 87.90
53	food-deprived (15 MIN) vs non-stressed (	12.00	0.7492	P > 0.05	-61.90 to 85.90
54	food-deprived (15 MIN) vs stressed (30 M	-5.000	0.3121	P > 0.05	-78.90 to 68.90
55	food-deprived (15 MIN) vs food-deprived (	14.00	0.8740	P > 0.05	-59.90 to 87.90
56	non-stressed (30 MIN) vs stressed (30 MI	-17.00	1.061	P > 0.05	-90.90 to 56.90
57	non-stressed (30 MIN) vs food-deprived (	2.000	0.1249	P > 0.05	-71.90 to 75.90
58	stressed (30 MIN) vs food-deprived (0 MI	19.00	1.186	P > 0.05	-54.90 to 92.90

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 6, STEP TEST				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	8			
8	F	211.4			
9	R squared	0.9585			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	8.783			
13	P value	0.2686			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	24270	7	3467	
19	Residual (within columns)	1050	64	16.40	
20	Total	25320	71		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NSR(L) vs NSR(R)	-20.56	15.23	P < 0.001	-26.54 to -14.57
24	NSR(L) vs SR(L)	-0.9259	0.6859	P > 0.05	-6.914 to 5.062
25	NSR(L) vs SR(R)	-31.85	23.59	P < 0.001	-37.84 to -25.86
26	NSR(L) vs NSNR(L)	0.7778	0.5762	P > 0.05	-5.210 to 6.766
27	NSR(L) vs NSNR(R)	-43.19	31.99	P < 0.001	-49.17 to -37.20
28	NSR(L) vs SNR(L)	-4.556	3.375	P > 0.05	-10.54 to 1.433
29	NSR(L) vs SNR(R)	-44.74	33.14	P < 0.001	-50.73 to -38.75
30	NSR(R) vs SR(L)	19.63	14.54	P < 0.001	13.64 to 25.62
31	NSR(R) vs SR(R)	-11.30	8.368	P < 0.001	-17.28 to -5.308
32	NSR(R) vs NSNR(L)	21.33	15.80	P < 0.001	15.35 to 27.32
33	NSR(R) vs NSNR(R)	-22.63	16.76	P < 0.001	-28.62 to -16.64
34	NSR(R) vs SNR(L)	16.00	11.85	P < 0.001	10.01 to 21.99
35	NSR(R) vs SNR(R)	-24.19	17.92	P < 0.001	-30.17 to -18.20
36	SR(L) vs SR(R)	-30.93	22.91	P < 0.001	-36.91 to -24.94
37	SR(L) vs NSNR(L)	1.704	1.262	P > 0.05	-4.284 to 7.692
38	SR(L) vs NSNR(R)	-42.26	31.30	P < 0.001	-48.25 to -36.27
39	SR(L) vs SNR(L)	-3.630	2.689	P > 0.05	-9.618 to 2.359
40	SR(L) vs SNR(R)	-43.81	32.46	P < 0.001	-49.80 to -37.83
41	SR(R) vs NSNR(L)	32.63	24.17	P < 0.001	26.64 to 38.62
42	SR(R) vs NSNR(R)	-11.33	8.395	P < 0.001	-17.32 to -5.345
43	SR(R) vs SNR(L)	27.30	20.22	P < 0.001	21.31 to 33.28
44	SR(R) vs SNR(R)	-12.89	9.548	P < 0.001	-18.88 to -6.901

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
45	NSNR(L) vs NSNR(R)	-43.96	32.57	P < 0.001	-49.95 to -37.97
46	NSNR(L) vs SNR(L)	-5.333	3.951	P > 0.05	-11.32 to 0.6548
47	NSNR(L) vs SNR(R)	-45.52	33.72	P < 0.001	-51.51 to -39.53
48	NSNR(R) vs SNR(L)	38.63	28.62	P < 0.001	32.64 to 44.62
49	NSNR(R) vs SNR(R)	-1.556	1.152	P > 0.05	-7.544 to 4.433
50	SNR(L) vs SNR(R)	-40.19	29.77	P < 0.001	-46.17 to -34.20

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 6, CYLINDER TOUCH				
3	One-way analysis of variance				
4	P value	0.0023			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	6.002			
9	R squared	0.3601			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	2.459			
13	P value	0.4828			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	3573	3	1191	
19	Residual (within columns)	6350	32	198.4	
20	Total	9924	35		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NSR vs SR	-9.900	2.108	P > 0.05	-27.90 to 8.104
24	NSR vs NSNR	-18.80	4.004	P < 0.05	-36.80 to -0.7959
25	NSR vs SNR	-26.70	5.686	P < 0.01	-44.70 to -8.696
26	SR vs NSNR	-8.900	1.895	P > 0.05	-26.90 to 9.104
27	SR vs SNR	-16.80	3.578	P > 0.05	-34.80 to 1.204
28	NSNR vs SNR	-7.900	1.682	P > 0.05	-25.90 to 10.10

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 6, CYLINDER WALL MOVE				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	11.12			
9	R squared	0.5105			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	7.713			
13	P value	0.0523			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	8889	3	2963	
19	Residual (within columns)	8523	32	266.4	
20	Total	17410	35		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NSR vs SR	-11.30	2.077	P > 0.05	-32.16 to 9.559
24	NSR vs NSNR	-22.90	4.209	P < 0.05	-43.76 to -2.041
25	NSR vs SNR	-42.50	7.812	P < 0.001	-63.36 to -21.64
26	SR vs NSNR	-11.60	2.132	P > 0.05	-32.46 to 9.259
27	SR vs SNR	-31.20	5.735	P < 0.01	-52.06 to -10.34
28	NSNR vs SNR	-19.60	3.603	P > 0.05	-40.46 to 1.259

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 6, CYLINDER LAND				
3	One-way analysis of variance				
4	P value	0.0022			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	6.039			
9	R squared	0.3615			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	2.236			
13	P value	0.5248			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	2496	3	831.9	
19	Residual (within columns)	4408	32	137.8	
20	Total	6904	35		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NSR vs SR	-8.700	2.224	P > 0.05	-23.70 to 6.300
24	NSR vs NSNR	-17.50	4.473	P < 0.05	-32.50 to -2.500
25	NSR vs SNR	-21.60	5.521	P < 0.01	-36.60 to -6.600
26	SR vs NSNR	-8.800	2.249	P > 0.05	-23.80 to 6.200
27	SR vs SNR	-12.90	3.297	P > 0.05	-27.90 to 2.100
28	NSNR vs SNR	-4.100	1.048	P > 0.05	-19.10 to 10.90

		A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 6,OPEN FIELD DISTANCE RU				
3	One-way analysis of variance				
4	P value	0.0051			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	5.154			
9	R squared	0.3258			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	24.01			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	32050000	3	10680000	
19	Residual (within columns)	66320000	32	2072000	
20	Total	98370000	35		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NSR vs SR	1870	3.897	P < 0.05	30.08 to 3710
24	NSR vs NSNR	70.00	0.1459	P > 0.05	-1770 to 1910
25	NSR vs SNR	1970	4.105	P < 0.05	130.1 to 3810
26	SR vs NSNR	-1800	3.751	P > 0.05	-3640 to 39.92
27	SR vs SNR	100.0	0.2084	P > 0.05	-1740 to 1940
28	NSNR vs SNR	1900	3.959	P < 0.05	60.08 to 3740

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 6, INNER ZONE ENTRY				
3	One-way analysis of variance				
4	P value	0.0324			
5	P value summary	*			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	3.311			
9	R squared	0.2369			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	24.92			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	64.97	3	21.66	
19	Residual (within columns)	209.3	32	6.542	
20	Total	274.3	35		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NSR vs SR	1.556	1.825	P > 0.05	-1.713 to 4.824
24	NSR vs NSNR	1.667	1.955	P > 0.05	-1.602 to 4.936
25	NSR vs SNR	3.778	4.431	P < 0.05	0.5089 to 7.047
26	SR vs NSNR	0.1111	0.1303	P > 0.05	-3.158 to 3.380
27	SR vs SNR	2.222	2.607	P > 0.05	-1.047 to 5.491
28	NSNR vs SNR	2.111	2.476	P > 0.05	-1.158 to 5.380

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 6, %DOPAMINE DESTRUCTIC				
3	One-way analysis of variance				
4	P value	0.0004			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	8.115			
9	R squared	0.4321			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	0.7046			
13	P value	0.8721			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	4553	3	1518	
19	Residual (within columns)	5984	32	187.0	
20	Total	10540	35		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	NSR vs SR	-9.970	2.187	P > 0.05	-27.45 to 7.507
24	NSR vs NSNR	-19.73	4.328	P < 0.05	-37.21 to -2.253
25	NSR vs SNR	-30.27	6.641	P < 0.001	-47.75 to -12.79
26	SR vs NSNR	-9.760	2.141	P > 0.05	-27.24 to 7.717
27	SR vs SNR	-20.30	4.453	P < 0.05	-37.78 to -2.823
28	NSNR vs SNR	-10.54	2.312	P > 0.05	-28.02 to 6.937

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 7, ACTH				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	8			
8	F	7.246			
9	R squared	0.5530			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	24.84			
13	P value	0.0008			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	2199	7	314.1	
19	Residual (within columns)	1777	41	43.34	
20	Total	3976	48		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	MSR (BASAL) vs NSR (BASAL)	2.738	1.057	P > 0.05	-8.961 to 14.44
24	MSR (BASAL) vs MSNR (BASAL)	-8.433	2.992	P > 0.05	-21.17 to 4.300
25	MSR (BASAL) vs NSNR (BASAL)	-0.4048	0.1563	P > 0.05	-12.10 to 11.29
26	MSR (BASAL) vs MSR (15 MIN)	-17.17	6.387	P < 0.01	-29.31 to -5.026
27	MSR (BASAL) vs NSR (15 MIN)	-10.33	3.845	P > 0.05	-22.47 to 1.807
28	MSR (BASAL) vs MSNR (15 MIN)	-7.333	2.728	P > 0.05	-19.47 to 4.807
29	MSR (BASAL) vs NSNR (15 MIN)	-13.50	5.023	P < 0.05	-25.64 to -1.359
30	NSR (BASAL) vs MSNR (BASAL)	-11.17	4.098	P > 0.05	-23.48 to 1.141
31	NSR (BASAL) vs NSNR (BASAL)	-3.143	1.263	P > 0.05	-14.38 to 8.097
32	NSR (BASAL) vs MSR (15 MIN)	-19.90	7.685	P < 0.001	-31.60 to -8.206
33	NSR (BASAL) vs NSR (15 MIN)	-13.07	5.047	P < 0.05	-24.77 to -1.372
34	NSR (BASAL) vs MSNR (15 MIN)	-10.07	3.889	P > 0.05	-21.77 to 1.628
35	NSR (BASAL) vs NSNR (15 MIN)	-16.24	6.270	P < 0.01	-27.94 to -4.539
36	MSNR (BASAL) vs NSNR (BASAL)	8.029	2.945	P > 0.05	-4.284 to 20.34
37	MSNR (BASAL) vs MSR (15 MIN)	-8.733	3.098	P > 0.05	-21.47 to 4.000
38	MSNR (BASAL) vs NSR (15 MIN)	-1.900	0.6740	P > 0.05	-14.63 to 10.83
39	MSNR (BASAL) vs MSNR (15 MIN)	1.100	0.3902	P > 0.05	-11.63 to 13.83
40	MSNR (BASAL) vs NSNR (15 MIN)	-5.067	1.797	P > 0.05	-17.80 to 7.667
41	NSNR (BASAL) vs MSR (15 MIN)	-16.76	6.472	P < 0.01	-28.46 to -5.063
42	NSNR (BASAL) vs NSR (15 MIN)	-9.929	3.833	P > 0.05	-21.63 to 1.770
43	NSNR (BASAL) vs MSNR (15 MIN)	-6.929	2.675	P > 0.05	-18.63 to 4.770
44	NSNR (BASAL) vs NSNR (15 MIN)	-13.10	5.056	P < 0.05	-24.79 to -1.396

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
45	MSR (15 MIN) vs NSR (15 MIN)	6.833	2.542	P > 0.05	-5.307 to 18.97
46	MSR (15 MIN) vs MSNR (15 MIN)	9.833	3.659	P > 0.05	-2.307 to 21.97
47	MSR (15 MIN) vs NSNR (15 MIN)	3.667	1.364	P > 0.05	-8.474 to 15.81
48	NSR (15 MIN) vs MSNR (15 MIN)	3.000	1.116	P > 0.05	-9.141 to 15.14
49	NSR (15 MIN) vs NSNR (15 MIN)	-3.167	1.178	P > 0.05	-15.31 to 8.974
50	MSNR (15 MIN) vs NSNR (15 MIN)	-6.167	2.294	P > 0.05	-18.31 to 5.974

	Parameter	A
		Value
		Y
1	Table Analyzed	CHAPTER 7, CORT
2	Column B	NSR (BASAL)
3	vs	vs
4	Column F	NSR (15 MIN)
5		
6	Unpaired t test	
7	P value	0.0116
8	P value summary	*
9	Are means signif. different? (P < 0.05)	Yes
10	One- or two-tailed P value?	Two-tailed
11	t, df	t=3.083 df=10
12		
13	How big is the difference?	
14	Mean ± SEM of column B	150.3 ± 34.83 N=7
15	Mean ± SEM of column F	305.8 ± 34.09 N=5
16	Difference between means	-155.5 ± 50.44
17	95% confidence interval	-267.9 to -43.14
18	R squared	0.4874
19		
20	F test to compare variances	
21	F,DFn, Dfd	1.461, 6, 4
22	P value	0.7436
23	P value summary	ns
24	Are variances significantly different?	No

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 8, WEIGHTS				
3	One-way analysis of variance				
4	P value	0.0027			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	5.734			
9	R squared	0.3295			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	20.12			
13	P value	0.0002			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	12250	3	4085	
19	Residual (within columns)	24930	35	712.3	
20	Total	37180	38		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	MS RUNNERS vs MS NON RUNNERS	-48.00	5.687	P < 0.01	-80.22 to -15.78
24	MS RUNNERS vs CONTROL RUNNERS	-34.00	3.921	P < 0.05	-67.11 to -0.8942
25	MS RUNNERS vs CONTROL NON RUNI	-31.00	3.673	P > 0.05	-63.22 to 1.223
26	MS NON RUNNERS vs CONTROL RUNI	14.00	1.615	P > 0.05	-19.11 to 47.11
27	MS NON RUNNERS vs CONTROL NON	17.00	2.014	P > 0.05	-15.22 to 49.22
28	CONTROL RUNNERS vs CONTROL NO	3.000	0.3460	P > 0.05	-30.11 to 36.11

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 8, STEP TEST				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	8			
8	F	147.2			
9	R squared	0.9364			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	10.43			
13	P value	0.1654			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	14040	7	2005	
19	Residual (within columns)	954.0	70	13.63	
20	Total	14990	77		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	MSR (L) vs MSR (R)	-32.00	27.41	P < 0.001	-37.17 to -26.83
24	MSR (L) vs NSR (L)	-6.000	5.002	P < 0.05	-11.31 to -0.6890
25	MSR (L) vs NSR (R)	-17.00	14.17	P < 0.001	-22.31 to -11.69
26	MSR (L) vs MSNR (L)	-3.000	2.570	P > 0.05	-8.169 to 2.169
27	MSR (L) vs MSNR (R)	-33.00	28.27	P < 0.001	-38.17 to -27.83
28	MSR (L) vs NSNR (L)	0.0000	0.0000	P > 0.05	-5.169 to 5.169
29	MSR (L) vs NSNR (R)	-26.00	22.27	P < 0.001	-31.17 to -20.83
30	MSR (R) vs NSR (L)	26.00	21.68	P < 0.001	20.69 to 31.31
31	MSR (R) vs NSR (R)	15.00	12.51	P < 0.001	9.689 to 20.31
32	MSR (R) vs MSNR (L)	29.00	24.84	P < 0.001	23.83 to 34.17
33	MSR (R) vs MSNR (R)	-1.000	0.8566	P > 0.05	-6.169 to 4.169
34	MSR (R) vs NSNR (L)	32.00	27.41	P < 0.001	26.83 to 37.17
35	MSR (R) vs NSNR (R)	6.000	5.140	P < 0.05	0.8307 to 11.17
36	NSR (L) vs NSR (R)	-11.00	8.939	P < 0.001	-16.45 to -5.551
37	NSR (L) vs MSNR (L)	3.000	2.501	P > 0.05	-2.311 to 8.311
38	NSR (L) vs MSNR (R)	-27.00	22.51	P < 0.001	-32.31 to -21.69
39	NSR (L) vs NSNR (L)	6.000	5.002	P < 0.05	0.6890 to 11.31
40	NSR (L) vs NSNR (R)	-20.00	16.67	P < 0.001	-25.31 to -14.69
41	NSR (R) vs MSNR (L)	14.00	11.67	P < 0.001	8.689 to 19.31
42	NSR (R) vs MSNR (R)	-16.00	13.34	P < 0.001	-21.31 to -10.69
43	NSR (R) vs NSNR (L)	17.00	14.17	P < 0.001	11.69 to 22.31
44	NSR (R) vs NSNR (R)	-9.000	7.504	P < 0.001	-14.31 to -3.689

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
45	MSNR (L) vs MSNR (R)	-30.00	25.70	P < 0.001	-35.17 to -24.83
46	MSNR (L) vs NSNR (L)	3.000	2.570	P > 0.05	-2.169 to 8.169
47	MSNR (L) vs NSNR (R)	-23.00	19.70	P < 0.001	-28.17 to -17.83
48	MSNR (R) vs NSNR (L)	33.00	28.27	P < 0.001	27.83 to 38.17
49	MSNR (R) vs NSNR (R)	7.000	5.996	P < 0.01	1.831 to 12.17
50	NSNR (L) vs NSNR (R)	-26.00	22.27	P < 0.001	-31.17 to -20.83

	Parameter	A Value Y	B Data Set-B Y	C Data Set-C Y	D Data Set-D Y
1	Table Analyzed				
2	CHAPTER 8, CYLINDER TOUCH				
3	One-way analysis of variance				
4	P value	0.0185			
5	P value summary	*			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	3.802			
9	R squared	0.2458			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	0.1881			
13	P value	0.9795			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	4953	3	1651	
19	Residual (within columns)	15200	35	434.2	
20	Total	20150	38		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	MSR vs NSR	10.30	1.521	P > 0.05	-15.55 to 36.15
24	MSR vs MSNR	-19.80	3.005	P > 0.05	-44.96 to 5.359
25	MSR vs NSNR	-11.50	1.745	P > 0.05	-36.66 to 13.66
26	NSR vs MSNR	-30.10	4.446	P < 0.05	-55.95 to -4.251
27	NSR vs NSNR	-21.80	3.220	P > 0.05	-47.65 to 4.049
28	MSNR vs NSNR	8.300	1.260	P > 0.05	-16.86 to 33.46

		A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 8, CYLINDER MOVEMENT				
3	One-way analysis of variance				
4	P value	0.0026			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	5.743			
9	R squared	0.3299			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	14.74			
13	P value	0.0021			
14	P value summary	**			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	2799	3	933.0	
19	Residual (within columns)	5686	35	162.5	
20	Total	8485	38		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	MSR vs NSR	5.300	1.280	P > 0.05	-10.51 to 21.11
24	MSR vs MSNR	-16.40	4.069	P < 0.05	-31.79 to -1.011
25	MSR vs NSNR	-10.60	2.630	P > 0.05	-25.99 to 4.789
26	NSR vs MSNR	-21.70	5.240	P < 0.01	-37.51 to -5.889
27	NSR vs NSNR	-15.90	3.840	P < 0.05	-31.71 to -0.08926
28	MSNR vs NSNR	5.800	1.439	P > 0.05	-9.589 to 21.19

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 8, CYLINDER LANDING				
3	One-way analysis of variance				
4	P value	0.0049			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	5.117			
9	R squared	0.3049			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	24.35			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	1597	3	532.3	
19	Residual (within columns)	3641	35	104.0	
20	Total	5238	38		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	MSR vs NSR	2.800	0.8450	P > 0.05	-9.852 to 15.45
24	MSR vs MSNR	-13.20	4.093	P < 0.05	-25.51 to -0.8854
25	MSR vs NSNR	1.200	0.3720	P > 0.05	-11.11 to 13.51
26	NSR vs MSNR	-16.00	4.828	P < 0.01	-28.65 to -3.348
27	NSR vs NSNR	-1.600	0.4828	P > 0.05	-14.25 to 11.05
28	MSNR vs NSNR	14.40	4.465	P < 0.05	2.085 to 26.71

	Parameter	A Value	B Data Set-B	C Data Set-C
		Y	Y	Y
1	Table Analyzed			
2	CHAPTER 8, OPEN FIELD MOVEMENT			
3	One-way analysis of variance			
4	P value	0.0001		
5	P value summary	***		
6	Are means signif. different? (P < 0.05)	Yes		
7	Number of groups	4		
8	F	8.992		
9	R squared	0.4353		
10				
11	Bartlett's test for equal variances			
12	Bartlett's statistic (corrected)	4.001		
13	P value	0.2614		
14	P value summary	ns		
15	Do the variances differ signif. (P < 0.05)	No		
16				
17	ANOVA Table	SS	df	MS
18	Treatment (between columns)	81730000	3	27240000
19	Residual (within columns)	106000000	35	3030000
20	Total	187800000	38	
21				
22	Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
23	MSNR vs NSR	-3500	6.189	P < 0.001
24	MSNR vs NSNR	-3490	6.341	P < 0.001
25	MSNR vs MSR	-1810	3.288	P < 0.05
26	MSR vs NSR	-1690	2.989	P > 0.05
27	MSR vs NSNR	-1680	---	P > 0.05
28	NSNR vs NSR	-10.00	---	P > 0.05

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	CHAPTER 8, REARS				
3	One-way analysis of variance				
4	P value	0.8937			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	4			
8	F	0.2029			
9	R squared	0.01709			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	9.104			
13	P value	0.0279			
14	P value summary	*			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	26.92	3	8.974	
19	Residual (within columns)	1548	35	44.23	
20	Total	1575	38		
21					
22	No post tests. P > 0.05				

	Parameter	A Value	B Data Set-B	C Data Set-C	D Data Set-D
		Y	Y	Y	Y
1	Table Analyzed				
2	% DOPAMINE DESTRUCTION				
3	One-way analysis of variance				
4	P value	0.0011			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	6.647			
9	R squared	0.3630			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	2.652			
13	P value	0.4484			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	3888	3	1296	
19	Residual (within columns)	6824	35	195.0	
20	Total	10710	38		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	MSR vs NSR	5.900	1.301	P > 0.05	-11.42 to 23.22
24	MSR vs MSNR	-17.00	3.850	P < 0.05	-33.86 to -0.1412
25	MSR vs NSNR	-16.50	3.737	P > 0.05	-33.36 to 0.3588
26	NSR vs MSNR	-22.90	5.048	P < 0.01	-40.22 to -5.579
27	NSR vs NSNR	-22.40	4.938	P < 0.01	-39.72 to -5.079
28	MSNR vs NSNR	0.5000	0.1132	P > 0.05	-16.36 to 17.36